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THE EFFECT OF ANTIDEPRESSANTS ON RODENT BRAIN GLUCOCORTICOID SYSTEMS

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A thesis submitted in partial fulfilment of the requirements of the Open University for the degree of Doctor of Philosophy

May 2001

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SUBMISSION DATE: 21 MAY 2001
AWARD DATE: 24 October 2001

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ABSTRACT

The aetiology of depression and mechanisms of action of antidepressant drugs continue to be evasive. Evidence suggests that alterations in brain corticosteroid receptors may be a crucial factor in these events. Previous measurements of corticosteroid receptors (CR) and CR mRNA in animal tissues following stress and/or antidepressant administration have not attempted to measure CR in intact animals using different antidepressants, in various tissues or in putative animal models of stress.

The following investigations were aimed at quantifying CR and plasma corticosterone concentrations using radioligand binding and high performance liquid chromatography or radio-immunoassays, respectively. Behavioural investigations conducted in animal models of stress were extended to study the effects of antidepressants.

Antidepressant administration to intact animals had various effects. Cortical CR were increased after 14 days of DMI; paroxetine induced a reduction in cortical CR after 14 days; no changes were observed following venlafaxine for up to 28 days. There were no significant changes in CR binding parameters in the hippocampus, striatum or hypothalamus following antidepressant administration. CR in the thymus were reduced following paroxetine.

No significant effects of olfactory bulbectomy or antidepressant administration were observed on CR binding parameters. However, significant increases in locomotor activity were observed in bulbectomised rats, which were attenuated by chronic, antidepressant treatment.

In a putative stress model involving chronic exposure of mice to predator odour, no significant effects were observed on locomotor activity following predator exposure and/or antidepressant administration. Sucrose intake was decreased, representing a possible anhedonic response to chronic predator stress. Predator stress and/or antidepressant administration had no significant effects on cortical or hippocampal CR binding or plasma corticosterone concentrations.

The results of these investigations demonstrate that CR alterations observed in stress/depression are not reflected in the chosen animal models. CR changes following chronic antidepressant administration, though forming part of a biochemical cascade, are not likely to constitute a common mechanism of action for antidepressant drugs.

To

Mrs. Pushpa Maurya

&

the late Professor Rajnarayan Maurya

ACKNOWLEDGEMENTS

I am indebted to Dr. A.E. Theodorou for giving me the inspirational opportunity to work on this project and for all of his guidance, enthusiastic supervision and infinite support over the years. My sincerest gratitude also to Professor R.W. Horton for his earnest encouragement and advice on all the work involved in producing this thesis.

I would like to thank Professor B.E. Leonard for providing the opportunity to work in the Department of Pharmacology, University College Galway. Dr. J.P. Kelly must be especially thanked for his efforts, advice and guidance during my time at UCG-and also for conducting the open field/dosing/surgical procedures in the OB study. Thanks also to Andrew Harkin and Thomas O'Connor, from whom I learned an enormous amount.

Many thanks to the NESCOT technicians Jenny, Gill, Christine, Gloria, for their willingness to help. Special thanks must go to Dr. Fazal Oozeer for conducting the plasma corticosterone measurements using the HPLC (especially the naïve studies) but especially his bizarre advice; Salim Nagi for his assistance in teaching me the binding assays; Andrew Walden for his help with the chemistry and his patience; Bert Smith and particularly Maureen Peters for their good humour and efficiency during the animal model studies. Thanks also to Mike Grimshaw, as Head of the Dept. of Applied Sciences (NESCOT), for financial support and encouragement.

Most gratitude goes to my family Mrs. Pushpa Maurya and Ms. Karishma Maurya for encouraging, financing and supporting this endeavour and enabling it to happen. An enormous amount of my gratitude must go to Liz Bate, to whom I owe my sanity. Finally, many thanks to Anuj Shah, for providing the encouragement required to complete this thesis by reminding me that there is life outside pharmacology.

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LIST OF ABBREVIATIONS

ACh	acetylcholine
ACTH	adrenocorticotrophic hormone
ADX	bilateral adrenalectomy
AGP	α-1 acid glycoprotein
ANOVA	analysis of variance
AP-1	activating protein-1
AVP	arginine vasopressin
BDNF	brain derived neurotrophic factor
BNST	bed nucleus of the stria terminalis
BSA	bovine serum albumin
cAMP	3', 5' cyclic adenosine monophosphate
CBG	corticosteroid binding globulin
CCK	cholecystokinin
CMS	chronic mild stress
CR	corticosteroid receptors
CREB	cAMP response element binding protein
CRF	corticotropin releasing factor
CSF	cerebrospinal fluid
DA	dopamine
DMI	desmethylimipramine
DNA	deoxyribonucleic acid
DPM	disintegrations per minute
DST	dexamethasone suppression test
ECT	electroconvulsive therapy
GH	growth hormone

GABA	γ-aminobutyric acid
GR	glucocorticoid receptors
GRE	glucocorticoid response element
HPA-axis	hypothalamic-pituitary-adrenocortical axis
HPLC	high performance liquid chromatography
HRE	hormone response element
ICSS	intracranial self stimulation
IL-1β	interleukin-1β
IL-6	interleukin-6
INF-α	interferon-alpha
MAOI	monoamine oxidase inhibitor
MHPG	3-methoxy-4-hydroxyphenylethylene glycol
MR	mineralocorticoid receptors
mRNA	messenger ribonucleic acid
NA	noradrenaline
NARI	noradrenaline reuptake inhibitors
NK cells	natural killer cells
NMDA	*N*-methyl-*D*-aspartate
NSB	non-specific binding
OB	olfactory bulbectomy
OFT	open field test
POMC	pro-opiomelanocortin
POS	predator odour stress
PTSD	post traumatic stress disorder
PVN	paraventricular nucleus
REM	rapid eye movement

RIA	radioimmunoassay
SB	specific binding
SCN	suprachiasmatic nucleus
SO	sham operated
SON	supraoptic nucleus
SSRI	selective serotonin reuptake inhibitor
TB	total binding
TCA	tricylic antidepressant
TNF-α	tumour necrosis factor-alpha
5-HT	serotonin
5-HIAA	5-hydroxyindole acetic acid
6-OHDA	6-hydroxydopamine
VTA	ventral tegmental area

CHAPTER 1

INTRODUCTION

1.1. Characteristics and origins of depression

Affective disorders are an important psychiatric problem. Primary features of these disorders are characterised by mood disturbances of either a depressed or elated nature, these being connected with an extensive range of cognitive, physiological and interpersonal impediments. Characteristic clinical symptoms of major depression include a pervasive dysphoric mood, generalised loss of drive, anhedonia, psychomotor retardation/agitation, diminished appetite, fatigue/lethargy, insomnia/hypersomnia and recurrent pessimistic or suicidal thoughts (Caldecott-Hazard *et al*, 1991a; 1992). The manifestations of depressive symptoms can range from brief, mild, downward mood swings to more extreme conditions requiring medical treatment and subsequent hospitalisation or even fatality due to suicide.

The taxonomy of mood disorder symptoms is complicated and diagnostic criteria associated with these states are classified in both the "Diagnostic and Statistical Manual of Mental Diseases" of the American Psychiatric Association (DSM-III, DSM-III-R, DSM-IV) and the World Health Organisation's "International Classification of Diseases" (ICD-10). The DSM divides depressive disorders broadly into the categories of unipolar (consisting of depressive episodes only) and bipolar (characterised by depressive episodes with periods of mania). Mood disorders may also be classified as primary or secondary depending on the absence or presence of other pre-existing psychiatric disorders. Reactive, or neurotic depression is found to arise in response to an identifiable external cause and is more easily treated than endogenous (psychotic) depression. The endogenous form of depression generally has an acute onset, arises from no apparent cause, can be accompanied by delusions and hallucinations and does not respond to attention and reassurance.

Major depression has the highest incidence of all mental health disorders with up to 20% of the population suffering depressive symptoms at any given time in the United States and England (Gold *et al*, 1988). Epidemiological reports have noted an increased prevalence of hospitalised patients with depression since 1950 and a growing risk of depression among young adults, adolescents and children. The life-time risk for a single first unipolar episode is 3-4% for men and 5-10% for women (5-12% for men and 10-20% for women in the United States), with increased chances (60-75%) of repeated episodes following recovery from the first major depressive episode (Caldecott-Hazard *et al*, 1991a; Nemeroff, 1998). Studies in industrialised nations indicate that approximately 2-3% of the population is hospitalised or seriously impaired by affective disorders at any given time. Patients diagnosed with major depression are also at increased risk from co-morbidity with substance abuse and anxiety disorders (Hale, 1997). A strong link also exists between depression and suicide, with 15% of depressed patients committing suicide (Grothe *et al*, 1992).

Although cognitive and electro-convulsive therapies are effective in treating some types of depression, the contemporary treatment of major depression is dominated by drug therapy. The pharmacological treatment of depression utilises a number of different classes of drugs that act via various mechanisms e.g. the monoamine oxidase inhibitors (MAOIs), tricyclic antidepressants (TCAs), selective serotonin reuptake inhibitors (SSRIs) and noradrenaline reuptake inhibitors (NARIs). Approximately 80% of patients respond to pharmacological and psychological therapy, either alone or combined. In the absence of continuous antidepressant treatment, more than 80% of patients will have repeated episodes within 2 years - this figure drops to 20% with antidepressant treatment (Frank *et al*, 1990). The extensive and recurrent nature of depressive disorders requires therapies that would benefit many people, offering a

discernible improvement in quality of life. However, approximately 15% of patients are poor or non-responders to pharmacotherapy and adverse effects with many antidepressant medications can be severe. The reduction of adverse effects from existing antidepressant medications and development of novel treatments is therefore imperative in the attempt to alleviate depressive symptoms.

It appears that, with its potentially severe, incapacitating nature and characteristic physical and biochemical changes, depression must be regarded as residing outside the normal, healthy human condition and therefore as a pathological state. Research into the aetiology and treatment of affective disorders is therefore warranted by virtue of their intensity, pervasiveness, persistence and interference with physiological and social functioning.

The origins of depressive symptoms are as yet unknown. There are a number of psychodynamic and cognitive theories of depression, focusing on childhood exposure to hypercritical, exploitative, emotionally unresponsive environments that can lead to the emergence of poorly integrated emotions and negative and distorted thinking in later life. In the last 30 years however, multidisciplinary approaches to the study of major mental disorders have provided evidence that major depression does in fact, possess biological components and is not, as previously thought, a functional disorder arising solely from a poor childhood environment or psychosocial stressors.

Many lines of evidence confirm the heritability of symptoms and therefore the involvement of genetically predisposing factors in depressive illness (reviewed in Gold *et al*, 1988). Several other possible biological precipitants of depression have been identified including hypothyroidism, adrenocortical hyperactivity, and effects due to

oestrogen, which may account for increased depression in women (Leibenluft, 1998). There is also evidence to suggest that seasonal changes may precipitate depressive symptoms (as in seasonal affective disorder, SAD) and the implication of viral infections has not been eliminated as causes of symptoms of depression (Mestel, 1997).

Many alterations in monoamine neurotransmitter functioning have been observed following the onset of depressive symptoms leading to theories that altered monoamine systems may be the primary cause of depression. An extensive body of evidence implicating the immune system with depression has also recently lead to an immunological hypothesis for depression. A factor which is of great importance in the development of the depressive syndrome is the prolonged exposure to various psychological and social stressors, which ultimately results in an impaired general coping strategy. This is reflected in the altered status of the endocrine system in depressed patients thus also creating an endocrinological theory of depression.

Though the origins of depression remain to be established, various proposed theories concerning the pathology of depression are briefly reviewed in the following sections.

1.2. Neurochemistry of depression

The first major hypotheses to address the biological basis of affective disorders were the catecholamine and indoleamine deficiency hypotheses proposed by Schildkraut (1965), Bunney & Davies (1965) and Coppen (1967). The combination of these hypotheses comprises the **monoamine hypothesis of depression**, which postulates that depression is causally related to decreased activity of the neurotransmitters noradrenaline (NA), 5-hydroxytryptamine (5-HT) and dopamine (DA) at certain central neuronal synapses.

Support for the monoamine hypothesis came from the observations that reserpine and tetrabenazine precipitated depressive symptoms in some hypertensive patients by depleting monoamines. Symptoms were alleviated by treatment with the antitubercular compound, iproniazid, a monoamine oxidase inhibitor (MAOI) from which a series of clinically useful MAOIs were developed. The tricyclics (TCAs), which restored monoamines to normal concentrations by inhibiting NA and 5-HT uptake, also alleviated symptoms of depression.

Reduced concentrations of the major NA, 5-HT and DA metabolites, MHPG (3-methoxy-4-hydroxyphenylethylene glycol), 5-HIAA (5-hydroxyindole acetic acid) and HVA (homovanillic acid) in CSF, urine and plasma from depressed patients were found to return to normal concentrations following antidepressant treatment (reviewed by Willner, 1987b). Other studies however reported an increased activation of the locus-coeruleus-NA system and increased NA and MHPG concentrations in depressed patients (Kelly & Cooper, 1998), these being consistently reduced following antidepressant therapy. Post mortem investigations of α_1 adrenoceptors have yielded conflicting results but generally do not demonstrate any alterations. Higher numbers of cortical α_2 adrenoceptors have been reported in post mortem tissue from depressed suicides. Blunted growth hormone responses to clonidine also indicate decreased responsiveness of the α_2-adrenoceptor system in depression. Post mortem human brain studies of β-adrenoceptor binding have yielded conflicting results (reviewed in Willner, 1987b). Peripheral β-adrenoceptor studies have demonstrated reduced β-adrenoceptors on leucocytes and/or lower cAMP production in response to β agonists.

A low central 5-HT turnover is generally characteristic though the complexity of 5-HT systems makes it difficult to simplify the specific role of 5-HT in depressive disorders. 5-HT uptake into platelets is consistently lower in depressed patients than in controls. An underactivity of the 5-HT system in depression is also indicated by blunted prolactin/growth hormone release in response to L-tryptophan. A blunted prolactin response to the 5-HT releaser fenfluramine and 5-HT uptake inhibitor clorimipramine also supports this. Post mortem studies in depressed patients have also consistently shown no differences in 5-HT and 5-HT_{1A} binding sites between depressed and control values. However, several studies have reported an increased density of 5-HT_{2A} binding sites in the frontal cortex of suicide victims (Caldecott-Hazard *et al*, 1991b; Hrdina *et al*, 1993).

The involvement of DA in depression is unclear and DA concentrations appear to follow NA alterations indicating that the former may result from the latter. Platelet studies suggest that there may be a defect of dopamine uptake in depression. Neuroendocrine challenge tests, investigating prolactin and growth hormone release, have yielded inconsistent results (see reviews by Van Praag, 1982; Willner, 1987b; Ackenheil, 1990; Brown & Gershon, 1993).

It soon became apparent that a wide range of neurotransmitters may be implicated in the pathophysiology of depression. The "classical" monoamine hypotheses of depression were extended to include possible roles for various other neurotransmitter systems. It has also been postulated (based on data which indicate that cholinergic agonists can induce depressive symptoms) that patients with major depression possess a hyperresponsive cholinergic system, which contributes to the disturbance of REM sleep patterns (Janowsky *et al*, 1972; Hasey & Hanin, 1991). Plasma and CSF concentrations

of the major inhibitory neurotransmitter GABA are reported to be lower in depressed patients (Gold *et al*, 1988). Many tricyclic and atypical antidepressants are found to inhibit the histamine stimulated increase of cAMP concentrations in the brain; however the role of histamine in depression remains unclear (Sugrue, 1983).

The most apparent discrepancy in the neurotransmitter hypothesis however was the contrast between the delay in the onset of therapeutic action of antidepressants compared with their acute neurochemical effects. This and several other ambiguities in this hypothesis led to its subsequent reformulation to the **"neurotransmitter receptor sensitivity hypothesis" of depression**. This associated secondary adaptive responses in neurotransmitter receptor systems, induced by chronic (not acute) antidepressant treatment, with the delayed onset of action of antidepressant medication and is logically consistent with the monoamine/neurotransmitter hypothesis. Alterations in neurotransmitter receptors following antidepressant administration have been reviewed in depth by Sugrue (1983), Maj (1984) and Caldecott-Hazard *et al* (1991b) and are briefly summarised below.

Receptor binding studies in animals display contradictory data regarding α_1-adrenoceptor numbers following the administration of various antidepressant compounds (Menkes *et al*, 1983; Maj, 1984). Clonidine induced locomotor activity/aggression is found to be enhanced by repeated administration of some antidepressants suggesting increased α_1 adrenoceptor responses following antidepressant administration (U'Prichard *et al*, 1978). Electrophysiological studies also indicate increased sensitivity of α_1 adrenoceptors, as do peripheral studies in human subjects (see Sugrue, 1983).

Results of α_2-receptor binding studies have also been contradictory demonstrating increased, decreased and unaltered α_2 sites in various brain regions following antidepressant administration in animals (Schoffelmeer *et al*, 1984; Garcia-Sevilla *et al*, 1986). **The α_2-adrenoceptor desensitisation hypothesis** (reviewed by McNeal *et al*, 1986; Ackenheil *et al*, 1990) states that presynaptic α_2-adrenergic autoreceptors are supersensitive in depression and antidepressants act to desensitise them. This hypothesis is supported by the attenuation of clonidine-induced hypothermia by repeated imipramine and ECS in animal studies. Electrophysiological experiments also confirm the presence of subsensitive α_2-adrenoceptors in rat brain following chronic desipramine. The principal tool for investigating α_2-adrenergic receptor function in humans is growth hormone (GH) secretion induced by the α_2-agonist clonidine. Blunting of the GH response has been demonstrated in several studies involving depressed patients; this being normalised following antidepressant administration (Matussek *et al*, 1980; Siever *et al*, 1984). It has been suggested that the reduced responsiveness of α_2 receptors may be associated with the mechanism responsible for dexamethasone non-suppression (see section 1.4.3.i).

The **β-adrenoceptor downregulation proposal** (Vetulani & Sulser, 1975; Peroutka & Snyder, 1980, Gillespie *et al*, 1988) suggested that TCAs may act by down-regulating postsynaptic β-adrenergic receptors in the brain following observations that central β-adrenergic receptor density was reduced following prolonged antidepressant administration. This reduction in β-adrenoceptors is the most consistent finding following long term TCA, MAOI, atypical antidepressant and ECT administration (Racagni *et al*, 1983; Byerley *et al*, 1987; 1988). The administration of a variety of antidepressants to animals is also found to result in decreased β-adrenoceptor stimulated cAMP formation in the limbic forebrain and cortex or in peripheral tissues

in humans. Electrophysiological investigations indicate a reduction of rat brain β-adrenoceptor sensitivity following chronic antidepressant administration (Sugrue, 1983). This indicates β-adrenoceptor down regulation or, possibly an uncoupling of the receptor and its effector which, interestingly, does not occur in the absence of a serotonergic input (Brunello *et al*, 1982; Sulser *et al*, 1983)

The overall picture at 5-HT synapses following antidepressant administration is one of enhanced transmission. Investigations into 5-HT receptors are made difficult due to the large numbers of subtypes that exist and the results of many studies into antidepressant effects on 5-HT receptors are inconsistent (reviewed by Sugrue, 1983; Fuxe *et al*, 1983; Willner, 1985; Stahl, 1986). Generally, 5-HT_1 receptors are unaltered by chronic antidepressants (except MAOIs). The rat 'serotonin syndrome' and hypothermia in the mouse, both induced by 5-HT_{1A} receptor agonists and thought to be mediated by 5-HT_1 receptors, are attenuated by chronic TCA, MAOI and ECT administration. Cortical 5-HT_2 receptors are found to be down-regulated by the administration of various antidepressants (except SSRIs) suggesting that this action may relate to antidepressant effects (Peroutka & Snyder, 1980). Many behavioural and electrophysiological studies point to an increased sensitivity of postsynaptic 5-HT receptors in the rat brain following long term antidepressant administration; however a lack of correlation appears to exist between these and results of binding studies (Sugrue, 1983; Ackenheil, 1990).

No consistent DA receptor alterations have been suggested however, a decreased sensitivity of D_2 autoreceptors has been reported in some animal studies (reviewed by Kapur *et al*, 1992). Studies of the postsynaptic D_2 receptor have generally found no effects of chronic administration of various antidepressants (Peroutka & Snyder, 1980;

Willner *et al*, 1983a; 1983b). It has been suggested that, given the enhancement of dopaminergic responses, but unaltered DA turnover and pre- and postsynaptic receptors following antidepressant administration, that changes in D_2 responsivity may be induced via facilitatory alterations in D_1 receptors, or at sites distal to the receptors via the second messenger system (Kapur *et al*, 1992).

Acetylcholine and histamine do not appear to be consistently affected by antidepressant administration. There is a reported increase in density of muscarinic ACh receptors following chronic amitriptyline administration. However this observation may be due to the drug's high anticholinergic effects (McNeal *et al*, 1986).

Though $GABA_A$ receptors are blocked by many antidepressants, it is the $GABA_B$ receptor upon which recent attention has been focused. There are many discrepancies in the results of investigations of $GABA_B$ receptor binding following antidepressant administration with some studies demonstrating up-regulation and others showing down-regulation of $GABA_B$ receptors on multiple dosage of antidepressants (reviewed in Broekkamp *et al*, 1995).

These reformulated hypotheses of neurotransmitter involvement in depression are more viable as possible explanations of the pathophysiology of depression. The popularity of monoamine hypotheses for depression can be assessed by the fact that they have dominated the field for more than 30 years. However, these concepts have been further complicated by observations that various neurotransmitter systems are interactive and quite frequently, alterations in one neurotransmitter system are found to affect another. The **NA/5-HT link hypothesis** proposes that NA and 5-HT systems are neuroanatomically linked and affect each other at various sites on the neural axis – this

link possibly being at the level of the β-adrenoceptor (Gillespie *et al*, 1988). This interaction was proposed following observations that the down-regulation of cortical/limbic forebrain β_1-adrenoceptors after up to 3 weeks of DMI administration only occurs in the presence of an intact 5-HT system. The simultaneous alterations seen in various neurotransmitter systems in depression and following antidepressant administration indicate extensive interactions between systems. Connections exist between α_1, α_2, β_1-adrenoceptors, 5-HT_1 and 5-HT_2 receptors with influences on the dopaminergic system, and acetylcholine (reviewed by Ackenheil, 1990). Chronic administration of NMDA receptor antagonists also results in β-adrenoceptor down-regulation and adaptations of the NMDA receptor complex (Paul *et al*, 1994; Skolnick *et al*, 1996) suggesting a more fundamental role for these receptors in the pathophysiology of depression.

The interaction of a wide range of neuromodulators (such as neuropeptides and prostaglandins) with components of various neurotransmitter systems also means that "classical" hypotheses of depression are being extended to include possible roles for the benzodiazepine receptor (File, 1996) and more recently, immunological factors such as cytokines and positive acute phase proteins (Connor & Leonard, 1998), neuroactive peptides such as substance P, cholecystokinin (CCK), corticotrophin-releasing factor (CRF) and steroids (Murphy, 1991; Kramer *et al*, 1998; Rupprecht & Holsboer, 1999; Griebel, 1999). Observations that peptides and 'classical' neurotransmitters can co-exist in the same neurone also suggest it is possible that antidepressants may owe their activity to an ability to modulate such inter-relationships. The presence of peptides and especially immunological factors in the same cell, combined with the pronounced effects of stress/depression on immunity has

also led to the formulation of an immunological hypothesis for depression. This is summarised briefly in the next section.

1.3. Immunology of depression

Widely reported immunological alterations in depressed patients have also led to the recent proposal for a **macrophage hypothesis of depression.** This suggests that depressive symptoms arise as a consequence of abnormally secreted cytokines and positive acute phase proteins from macrophages which subsequently lead to hypersecretion of CRF (corticotropin releasing factor), adrenocorticotrophic hormone (ACTH), prolactin and cortisol in depression (reviewed in Ur, 1992). Further support for this hypothesis comes from observations that administration of various immunological challenges in animals produces alterations in behaviour, monoamine neurotransmitter and endocrinological systems that are similar to those seen in depression (reviewed in Connor, 1998).

Some studies have demonstrated impaired mitogen stimulated lymphocyte proliferation, zymosan-induced neutrophil phagocytosis and natural killer (NK) -cell toxicity in depressed patients. Decreased lymphocyte, neutrophil and monocyte counts have also been reported (O'Neill & Leonard, 1986; reviewed in Connor, 1998).

Depression is also associated with increased serum levels of interleukin-1β and –6 (IL-1β, IL-6), tumour necrosis factor-alpha (TNF-α), interferon-alpha (INF-α) and α_1-acid glycoprotein (Maes *et al*, 1993). IL-1β, IL-6 and α_1-acid glycoprotein levels have been reported to return to normal in patients who responded to chronic antidepressant therapy (Connor, 1998). Increased numbers of T-helper (CD4+), T-memory (CD4+, CD44RO+) and activated T-cells (CD25+, HLA-DR+) in serum from depressed

patients also indicates immunological activation (Maes *et al*, 1995). Song *et al* (1994) have also demonstrated raised plasma concentrations of the positive acute phase proteins (haptoglobin, α_1.antitrypsin, α_1 and α_2 macroglobulin) in depressed patients, in addition to increased cortisol and serum complement levels and decreased negative acute phase proteins.

There appears to be a large degree of interplay between components of the nervous, immune and endocrine systems. The hypothalamic-pituitary-adrenocortical (HPA) axis is the key player in endocrinological responses to stress. A variety of consistently observed endocrinological and HPA axis changes have been observed in depression culminating in an HPA-axis based hypothesis for the origin of depressive syndromes which forms the basis for the majority of work in this thesis. This hypothesis is discussed in more detail in the following sections which deal with the physiology and regulation of the HPA axis before moving on to the HPA dysfunction observed in depression and effects of various pharmacological treatments.

1.4. Endocrinology and depression

1.4.1. The HPA axis

The HPA axis plays a fundamental adaptive role in the face of stress on mammalian systems primarily via the secretion of corticosteroids. Hypothalamic neuropeptides, CRF, a 41 amino acid peptide and arginine-vasopressin (AVP) are synthesised by neurones of the paraventricular nucleus (PVN) which are innervated by nerve fibres containing various neurotransmitters or neuropeptides (Swanson *et al*, 1983). CRF and AVP, along with oxytocin and NA act as ACTH secretagogues. The production and secretion of oxytocin occurs in the supraoptic nucleus (SON) of the hypothalamus and NA is derived mainly from brainstem catecholaminergic nuclei, however it is mainly CRF that subsequently stimulates the release of adrenal corticosteroids. CRF and AVP are released from the median eminence into the hypophyseal circulation from where they travel to the anterior pituitary and are thought to act in a synergistic fashion on pituitary corticotropes to stimulate the release of ACTH (along with other pro-opiomelanocortin derived peptides, including endorphins). The secretion of cortisol from the adrenal gland is driven by ACTH, which stimulates cholesterol uptake and synthesis in the adrenal cortex (Figure 1.4.1.i; reviewed by Stokes, 1987; de Kloet, 1991). The adrenal steroids are synthesised from step-wise enzymatic modifications of cholesterol from the blood or from acetate within the adrenocortical cells.

Cortisol is the major corticosteroid in humans with small amounts of corticosterone synthesised as a by-product. Corticosterone is produced as the major corticosteroid in the rat. Small amounts of mineralocorticoids, androgens and oestrogen are also produced as by-products of corticosteroid synthesis (reviewed in Dallman *et al*, 1987).

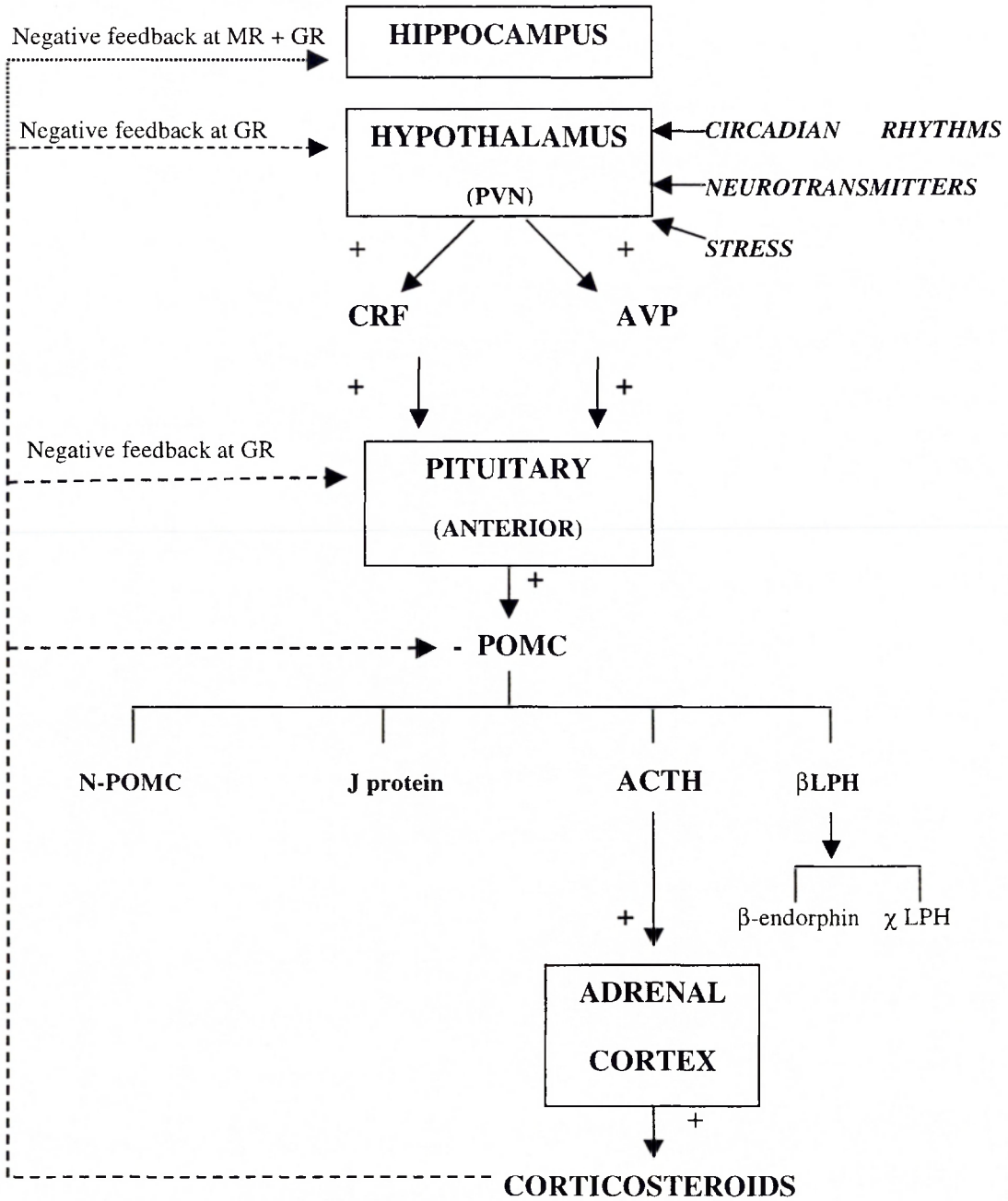


Figure 1.4.1.i. Schematic diagram showing structure and regulation of the hypothalamic-pituitary-adrenocortical axis (HPA) axis. The hypothalamic factors, CRF (corticotropin releasing factor) and AVP (arginine vasopressin) act synergistically to induce POMC-derived ACTH (adrenocorticotropin) secretion from the anterior pituitary. ACTH stimulates the secretion of corticosteroids from the adrenal cortex, which in turn, can elicit a negative feedback effect on the anterior pituitary and hypothalamus. Inhibitory feedback effects of the peripheral effector hormones are mediated through a binary corticosteroid receptor system within the brain; GR (glucocorticoid receptors) and MR (mineralocorticoid receptors).

1.4.2. Corticosteroid receptors

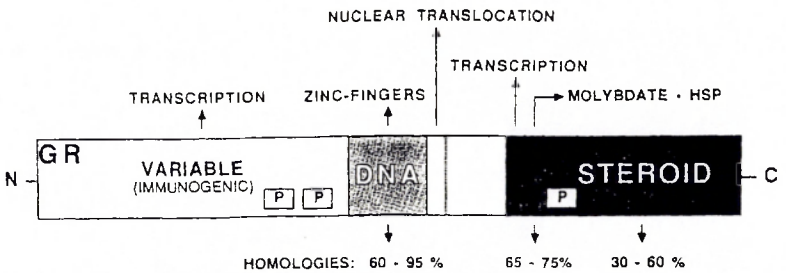
Biologically active free corticosteroids exert their effects through their genomic actions on two types of corticosteroid receptors (CR), the mineralocorticoid receptor (MR) or type I CR and the glucocorticoid receptor (GR) or type II CR. Both types of CR are members of the steroid hormone receptor family (also including androgen and progesterone receptors) which are all similar with regards to their structure and organisation into discrete functional domains. In addition to highly conserved domain structures within this receptor group, MR and GR display a particularly high degree of sequence homology (Figure 1.4.2.i.).

The carboxy-terminal domain contains the ligand/heat shock protein (Hsp) binding site and receptor dimerisation, transactivation and nuclear localisation functions. The sequences between the steroid and DNA binding regions are known as the hinge region and are thought to contribute to the nuclear translocation, transcription and steroid binding activities of the receptor molecule. The DNA binding domain is located roughly in the centre of the molecule and directs dimerisation, sequence-specific DNA binding of the receptor and also contains a nuclear localisation signal. This cysteine-rich region consists of highly conserved peptide projections containing two Zn^{2+} motifs that promote the interaction of CR with target enhancers (see Figure 1.4.2.i, Schwabe & Rhodes, 1991). Each zinc finger is important for high affinity binding to target DNA sequences and are involved with the initial contact and correct orientation of residues that enable DNA-protein interaction and/or receptor dimerisation. Structural studies indicate that the first (N-terminal) finger plays a more important role in sequence specific DNA recognition. The interfinger region/C-terminal finger are required for prevention of non-specific binding to other steroid hormone responsive DNA elements and perhaps for activated steroid-receptor complex dimerisation (Bodine & Litwack,

1990; O'Malley & Tsai, 1992). The highly variable amino-terminal domain largely has a modulatory function and regulates interactions with other transcription factors (Gehring, 1993; Funder, 1993; reviewed by Simons, 1994).

Figure 1.4.2.i Domain structure and homologies of human corticosteroid receptors

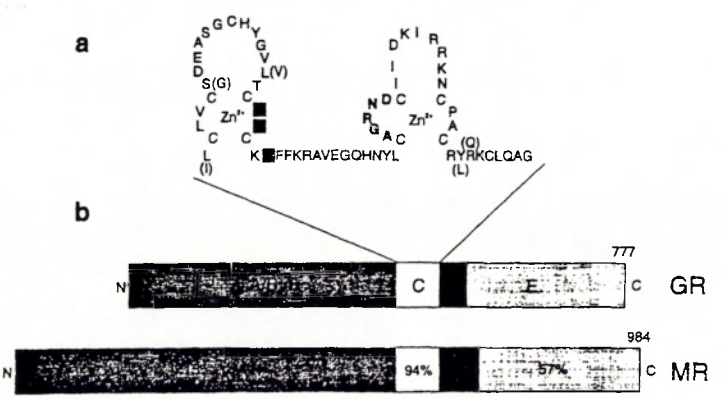
(1)



HUMAN RECEPTOR	CHROMOSOMAL LOCATION	AMINO ACID NUMBER	APPROXIMATE MOLECULAR WEIGHT	CELLULAR LOCATION (UNOCCUPIED)
MR	4	984	113,000	CYTOPLASM ?
PR	11	933	107,000	NUCLEUS
AR	X	918	100,000	NUCLEUS
GR	5	777	90,000	CYTOPLASM
ER	6	595	68,000	NUCLEUS

Domain structure and sequence homology of GR and steroid hormone receptors. Key; **N**, *amino terminus*; **C**, *carboxy terminus*; **HSP**, 90,000 *dalton heat shock protein*; **P**, phosphoserine residues; **GR**, glucocorticoid receptor; **MR**, mineralocorticoid receptor; **PR**, progesterone receptor; **AR**, androgen receptor; **ER**, eostrogen receptor; **VDR** vitamin D receptor; **THR**, thyroid hormone receptor; **RAR**, retinoic acid receptor; **AHR**, aromatic hydrocarbon receptor, Taken from Bodine & Litwack (1990).

(2)



a) Amino acid sequence of DNA binding domain of the human glucocorticoid receptor (GR and mineralocorticoid receptor (MR). Solid squares indicate P-box amino acids which determine sequence specificity and bold type indicates D-box amino acids which mediate dimerisation. **b**) Functional domains of human GR (777 amino acids) and MR (984 amino acids). A= modulatory region, B=DNA-binding region, C= ligand-binding region. Percentage sequence homology of the human MR relative to the human GR is shown. Diagram taken from Trapp & Holsboer (1996).

The two receptor systems represented by MR and GR, located in the brain as well as peripherally, have been identified based on their affinities for endogenous and synthetic corticosteroids. MR and GR display considerable sequence homology therefore share numerous ligands however, many of these produce very specific responses at these receptors. This suggests a dual action of the corticosteroid hormones in the control of brain function.

The type I MR has a dissociation constant (K_D) for corticosterone of 0.5-1nM and with relative steroid binding affinities of corticosterone ≥ aldosterone >> dexamethasone (a synthetic glucocorticoid). MR in the brain are found mostly in the CA3 area of the hippocampus and the septum. The mediation of tonic actions of corticosteroids is proposed as the primary function of MR as these are under most circumstances, extensively occupied by corticosteroids. Type II GR have a lower affinity for endogenous corticosteroids (K_D ~2-5nM) and the following rank order of steroid binding affinities, dexamethasone> corticosterone >> aldosterone (Moses *et al*, 1991). Type II GR exhibit a more ubiquitous distribution in the brain. They are present in the PVN and other hypothalamic nuclei, the limbic system (including the hippocampus – CA1 and CA2 pyramidal neurones, septum and amygdala), the cerebral cortex and many brainstem monoaminergic nuclei (Reul *et al*, 1985; Eekelen *et al*, 1987). Observations that these receptors become occupied concurrently with rising plasma corticosteroid concentrations following stress (Reul *et al*; 1987a; 1987b) suggest that GR primarily mediate feedback actions of corticosteroids aimed at switching off stress-activated brain mechanisms. The responsiveness of GR to varying plasma corticosteroid levels infers on them greater plasticity and capacity for autoregulation.

Corticosteroids exert and regulate neuronal excitability via several different, diverse mechanisms beginning with the determination of access of hormone to the receptors by corticosteroid binding globulin (CBG), and steroid metabolising enzymes such as 11β-hydroxysteroid dehydrogenase type I (abundant in the kidney) which results in location- and receptor-specific activation. Corticosteroids enter brain cells by diffusion through cell membranes and bind to intracellular receptors which form part of a cytoplasmic multiprotein complex consisting of one corticosteroid receptor molecule and several heat shock proteins (Hsp-90, Hsp-70, Hsp-56 and immunophilins). These proteins are necessary to enable the receptor to bind ligands with high affinity as ligand-independent dissociation of these proteins would prevent efficient receptor-ligand binding. The steroid binding activities of CR are also stabilised by complexes formed between thiol groups and sodium molybdate, which is thought to interact with cysteine residues of the receptor molecule (Bodine & Litwack, 1990).

The binding of corticosteroids results in dissociation of the receptor-Hsp complex, several phosphorylation stages, alteration in the conformation of the receptor protein and an activated receptor with increased affinity for nuclear DNA binding domains (Figure 1.4.2.ii). Seven major phosphorylation sites, many containing Ser-Pro sequences, have been identified in the mouse GR, all of which are located in the region N-terminal to the DNA binding domain. The steroid free CR is a phosphoprotein and steroid binding causes a 2-4 fold increase in phosphorylation which plays important roles in nuclear translocation, DNA binding, interactions with other proteins and transactivation - and can alter the activity/stability of the receptor protein in response to different signalling pathways (Weigel, 1996). Reduced cysteines are thought to be required for steroid binding and the conversion of the receptor to a DNA binding form.

Hormone-activated receptors undergo 'transactivation' which involves topological alterations that expose the DNA binding domain of the receptor. After nuclear translocation, activated receptors associate with specific DNA sequences (hormone response elements, HRE's) in regulatory regions of target genes to mediate transcriptional activity. Recent evidence suggests that MR/GR can bind to DNA sequences as homo- or heterodimers (Trapp *et al*, 1994). Heterodimerisation may form an important mechanism in regions in which MR and GR are co-localised (in major regulatory areas of the HPA axis) whereas it may only play a minor role in other target areas such as the liver where the MR concentrations are low. For example, in the hippocampus, the relative concentrations of the receptors and their ligands enables a definition of the proportion of each type of CR dimer - with its own DNA and transactivation properties, allowing a more finely tuned regulation of corticosteroid responses and more diverse hormone signalling processes (Trapp & Holsboer, 1996). The relative activation of MR/GR is an important factor which may synergistically determine changes in the excitability of such cells containing co-localised receptors (Simons, 1994; Zakon, 1998).

The mechanisms by which CR regulate transcription are as yet unclear and constitute an intensively pursued area of research. Activated CR dimers are thought to bind to the consensus sequence of a simple HRE comprised of 15 base pair palindrome 5'-GGTACA-nnn-TGTTCT-3'. Aside from activated-CR binding to DNA, little is known about the actual interaction of the receptor complex with chromatin. It is possible that the activated receptor-complex binds to nucleosomes which appear to contain HREs and the binding of the activated receptor complex disrupts this structure resulting in the opening of the DNA helix. This would enable protein transcription factors such as the CAAT-box and the TATA-box to bind to promoter regions of a corticosteroid-

responsive gene which may create a DNA-protein complex to which RNA polymerase II can bind and initiate mRNA synthesis (Bodine & Litwack, 1990).

A proposed mechanism by which activated CR complexes may alter nucleosome structure and activate other factors involves the demonstration that the receptor complex is associated with a protein kinase forming a complex capable of phosphorylating histones. In the presence of calcium, the receptor is phosphorylated on threonine residues by the protein kinase representing an 'autophosphorylation' reaction. This process may at least partly form the mechanism by which transcription is enhanced by corticosteroids (Bodine & Litwack, 1990).

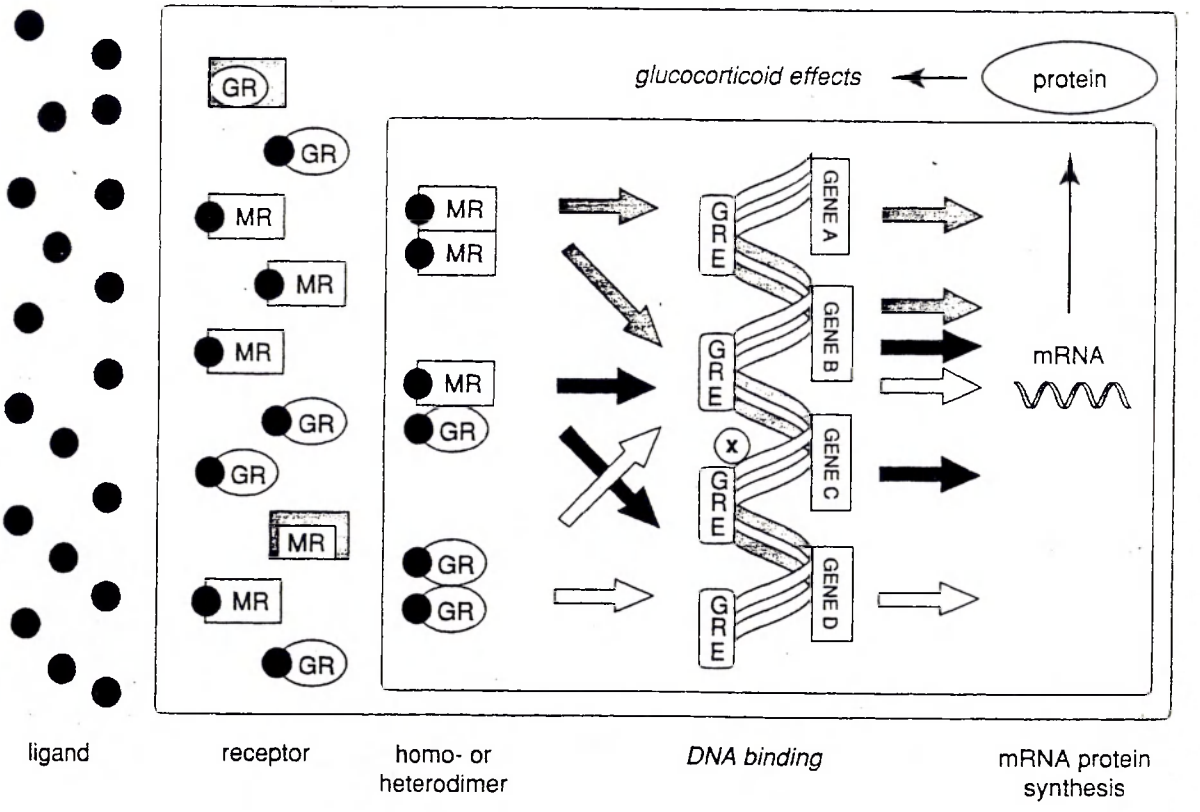
CR dimers may mediate different effects at composite HREs an example of which is 'pflG', a 25-nucleotide consisting of a low-affinity HRE and AP-1 (activating protein-1) binding site. The AP-1 site in this response element is capable of binding c-Fos and c-Jun (modulatory proto-oncogene products) homo- or heterodimers. These dimers are thought to form leucine 'zipper ' complexes which non-covalently bring together the domains of the CR in a functionally active form that binds to the HRE of DNA. The ratio of c-Fos and c-Jun homo- or hetero-dimers formed is also a mechanism through which receptor activity can be selected (Miner & Yamamoto, 1991). Activated GRs can block c-Jun-c-Fos-enhanced transcription from the composite GRE but activated MRs cannot, thus conferring specificity also at this stage of the interaction (Funder, 1993). It is thought that transcription may be induced via the steroid activated TAF-2 (tau2) sequences that are brought into proximity of the transcriptional machinery by a Jun-Fos-mediated associated with the HRE-bound binding domain. This regulatory 'cross-talk' may also occur between activated CR and other nuclear factors such as the CCAAT-box and CACCC-box binding proteins, nuclear factor-1, promoter selective

transcription factor, cAMP response element binding protein (CREB) and nuclear factor-κB (NF-κB), interleukin-6 which may be activated by various other agents (neurotransmitters and other hormones).

Transactivational interactions with other transcription factors, along with modulation of activity through second messenger-coupled membrane receptors, are also important for the net result of steroids on neuronal firing. However, it is clear that the complexities of steroid receptor regulation in neurones serve to produce numerous very specific effects. Direct transcriptional regulation by CR appears to be at the level of mRNA initiation and does not affect the rate of transcription - which appears to be more dependent on the cellular concentration of the steroid-receptor complex. In addition to their direct effects on transcription, corticosteroids can also exert indirect and post-transcriptional effects (Bodine & Litwack, 1990).

Activated CR complexes can also act as repressors of transcriptional activity. This is thought to be conducted via a 'steric hindrance' mechanism. In the case of the POMC gene in the anterior pituitary, the activated CR-complex inhibits transcription by binding to a negative HRE located within the promoter region of the POMC gene. This DNA binding site overlaps the binding site for a putative member of the COUP-transcription factor group thus the receptor may inhibit transcription by preventing the binding of a COUP-like transcription factor to its DNA recognition sequence. Similar mechanisms may exist for other corticosteroid-responsive genes (Bodine & Litwack, 1990). Following translocation and transcription (if it occurs) the steroid-receptor complex dissociates with the steroid being metabolised and the receptor being degraded down to amino acids or recycled back to a steroid-binding form.

Figure 1.4.2.ii. Model of corticosteroid receptor-activated gene expression.



Steroids enter the cell by passive diffusion where they bind to the cognate receptor, the glucocorticoid receptor (GR) or the mineralocorticoid receptor (MR). This binding leads to a conformational change in the receptor and its dissociation from the inhibitory hetero-oligomeric structure. After intranuclear translocation, the MR or GR homodimers or the corticosteroid receptor (CR) heterodimer is constituted depending on the relative concentrations of both receptors. The different CR dimers bind to glucocorticoid response elements (GREs) in the flanking regions of target genes with particular DNA binding kinetics. Binding of the GRE leads to mRNA production and protein synthesis. The unique transcriptional activities and DNA-binding kinetics of the CR dimers are represented by different coloured arrows. The use of different genes in this illustration indicates the idea that different corticosteroid dimers may regulate an overlapping set of genes and activate the expression of dimer-specific candidate genes. The regulation of certain genes by a specific CR dimer may predominantly depend on dimer specific, protein-protein interactions with other transcription factors that are represented in this model by a DNA-binding factor X. Diagram taken from Trapp & Holsboer (1996).

1.4.3. Regulation of the HPA axis

i) Feedback - Like all neuroendocrine axes, the HPA axis possesses a number of sites of inhibitory feedback in order to maintain an intrinsic homeostatic system (Figure 1.4.1.i). Feedback mechanisms of plasma corticosteroid regulate HPA activity under basal and stress conditions. When corticosteroid concentrations are high, CRF and ACTH production is suppressed thereby reducing corticosteroid secretion. Conversely, CRF and ACTH levels increase when corticosteroid levels are inappropriately low. CRF and ACTH are important control factors in HPA regulation producing activation of CNS function when centrally administered (Holsboer *et al*, 1985; Dunn *et al*, 1990).

Corticosteroid feedback can be conducted in a proactive (maintenance of basal HPA activity) or reactive (termination of stress-induced HPA adaptation) manner. Regulation is mediated by MR/GR and the HPA axis responds rapidly and transiently, via "fast feedback" mechanisms to acutely changing rates of plasma corticosteroids and in a "delayed" manner following chronic alterations of corticosteroid secretion (de Kloet, 1998).

Fast feedback sites are thought to be mainly located in the pituitary (Smelik *et al*, 1977) and hippocampus (McEwen *et al*, 1969; Sapolsky *et al*, 1984a; 1994b). This response operates over a time scale of minutes and is proportional to the rate of change in plasma corticosteroids. It can be tested in humans by the suppression of plasma ACTH and β-endorphin/β-lipotropin following an infusion of hydrocortisone (Young *et al*, 1991). It is thought that mechanisms for fast feedback may involve a direct antagonistic action of glucocorticoids on CRF binding to its pituitary receptors or antagonism of CRF-dependent cAMP generation in pituitary corticotrophs. *In vivo* studies however suggest that rapid feedback is probably mediated at

hippocampal/hypothalamic sites (Sapolsky *et al*, 1984b). NA input also appears to be necessary in this type of feedback process as experiments in rats involving pre-treatment of noradrenergic neurones with 6-hydroxydopamine (6-OHDA) demonstrate an inhibition of fast feedback effects of corticosterone on histamine-induced CRF synthesis (Stokes & Sikes, 1987). The neural pathways that mediate steroid feedback inhibition are not yet clear.

The delayed response is progressive, persists for as long as corticosteroid levels are elevated and may require adaptations at receptor level to enable the system to tolerate higher corticosteroid concentrations. Delayed feedback occurs over several hours and is proportional to the mean plasma corticosteroid concentration over that time. It can be tested in clinical practice by the suppression of HPA function by dexamethasone/CRF, a test that involves GR in the pituitary. The dexamethasone suppression test (DST) uses a 1-2mg dose of dexamethasone orally administered around 23.00 hours, which suppresses cortisol secretion. A large proportion (~60%) of depressed patients exhibit early escape from dexamethasone suppression when tested in the morning (Carroll *et al*, 1981; Coryell, 1990). Recent modifications to this test involve the administration of 100μg human CRF to subjects on the day of the test. In controls, release of ACTH and cortisol are prevented; however, in depressed patients, an increased secretion of ACTH and cortisol is observed (Heuser *et al*, 1994). Delayed feedback also involves corticosteroid action, mainly at hippocampal, hypothalamic and pituitary levels (Jacobson & Sapolsky, 1991; Dallman *et al*, 1992, Young *et al*, 1990).

The hippocampus exerts predominantly inhibitory influence on HPA responses to stress and several studies have demonstrated the inhibition of corticosteroid secretion following hippocampal stimulation. Experiments have also demonstrated that

adrenalectomised (ADX), hippocampectomised rats show circadian peak plasma ACTH levels similar to those in adrenalectomised rats not subjected to hippocampectomy. Reduced efficacy of dexamethasone suppression of the corticosteroid response in rats is also observed following damage to the hippocampus or fornix (Jacobsen *et al*, 1991). However, lesions to the hippocampus or fornix result in impaired dexamethasone suppression or reduced feedback sensitivity and not complete resistance to corticosteroid feedback. These data suggest that the primary function of the hippocampus is mediation of corticosteroid feedback regardless of whether or not it also contributes a tonic inhibitory influence in the face of fluctuating corticosteroid concentrations. It also appears that the hippocampus is not the only regulatory site of corticosteroid feedback activity or its elimination would result in total corticosteroid insensitivity.

Further studies have also implicated the pituitary and parvocellular PVN as sites of corticosteroid feedback. It is thought that different regions may be involved with regulation of various levels of corticosteroids via MR and GR thus mediating context-dependant feedback to circuits that are activated in a stressor-specific manner. It has been suggested that hippocampal MR are involved in the proactive maintenance of basal HPA activity throughout the circadian cycle whereas corticosteroid activation of hippocampal GR inhibits MR-mediated inhibition of the HPA axis. Parvocellular GR mediate reactive feedback inhibition aimed at terminating the HPA stress response (de Kloet, 1998).

ii) Circadian rhythms - Under basal conditions, regulation of the HPA axis occurs via circadian rhythms. The central mechanisms responsible for this are unknown but appear to be controlled by oscillators in the suprachiasmatic nucleus (SCN) and serotonergic innervations (Stokes & Sikes, 1987). There is also a circadian pattern in hippocampal 5-HT receptor mRNA (Holmes *et al*, 1997). The circadian rhythm of corticosteroid output roughly parallels the activity cycle, with plasma corticosteroid concentrations generally at their highest before waking and lowest before sleeping (Figure1.4.3.i.A). This cycle of corticosterone secretion is reversed in rats and mice (Figure 1.4.3.i.B and 1.4.3.i.C).

Basal HPA activity at the trough of the circadian cycle is thought to result from pituitary and adrenal secretions without hypothalamic inputs. This suggestion is based on observations that lesions of the basal hypothalamus, PVN or SCN do not decrease a.m. ACTH concentrations below their already low levels, but do prevent peak ACTH concentrations. Also basal corticosterone in ADX rats is indistinguishable from that of normal rats but peak plasma corticosterone is markedly reduced (Dallman *et al*, 1992). Regulation of HPA activity at the nadir of the cycle is achieved mostly by corticosteroid occupation of MR (de Kloet & Reul, 1987; Reul *et al*, 1987a). These receptors are thought to provide sufficient tonic control of HPA responses and under basal levels of corticosteroid output, are found to be ~25% occupied (Jacobsen, 1991).

HPA activity during the peak of the rhythm is thought to require hypothalamic inputs that may be driven by signals from the SCN. The increased levels of corticosteroids at this time involve the occupation of both MR and GR in feedback control of HPA function (Reul *et al*, 1987b). Under basal conditions, GR are ~10% occupied, with

occupation increasing with rising corticosteroid concentrations which, in turn, increase with varying degrees of stress (Jacobsen, 1991; Reul *et al*, 1985).

Underlying mechanisms of circadian corticosteroid output are thought to involve steady MR-controlled hippocampal excitatory output during low corticosteroid levels, which regulates GABA-ergic tone on PVN neurones. Activation of hippocampal GR as plasma corticosterone concentrations increase, inhibits MR-mediated inhibition of the HPA axis. GR appear to be more involved with regulating peak level and also stress induced corticosteroid concentrations. The hippocampus is proposed as the main site for mediating this procedure as i) it contains both types of CR ii) lesions of this region have demonstrated reductions in the diurnal changes in plasma corticosteroids, primarily by elevation of the nadir corticosteroid concentration toward that of the peak (De Kloet, 1987; Jacobsen *et al*, 1991; de Kloet, 1998).

Figure 1.4.3.i. Circadian rhythm of plasma cortisol/corticosterone secretion in humans (A) - taken from Dallman *et al*; 1992, rats (B) and mice (C)–taken from Shimuzu *et al*; 1983.

(A)

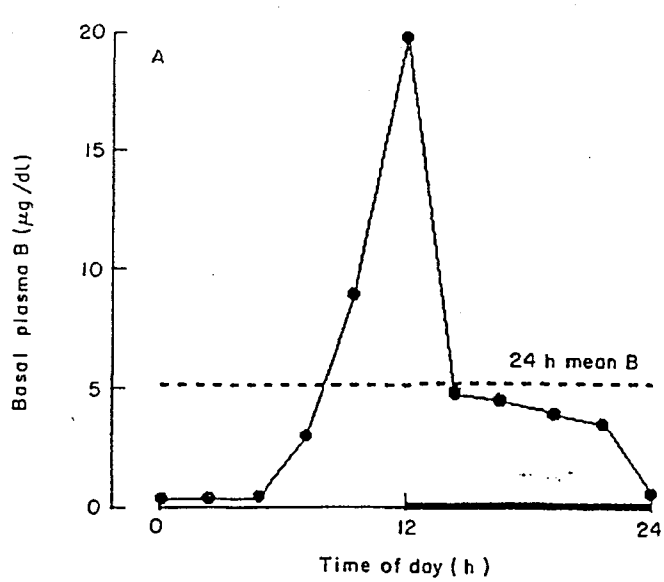
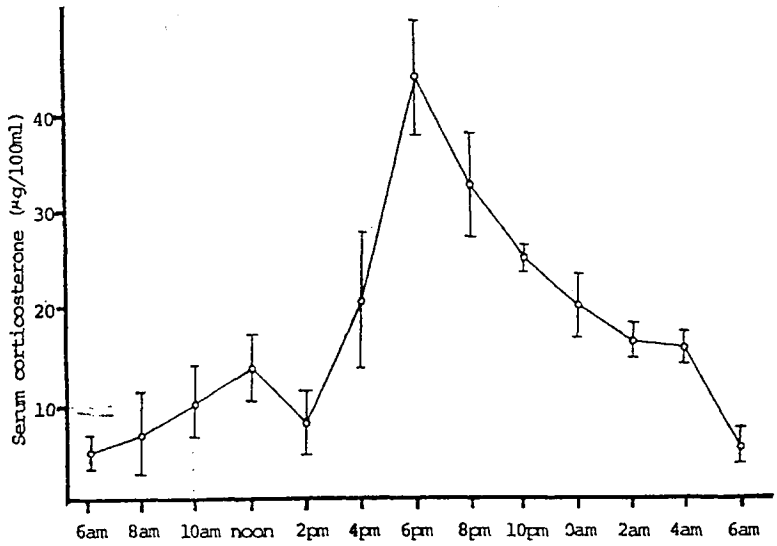
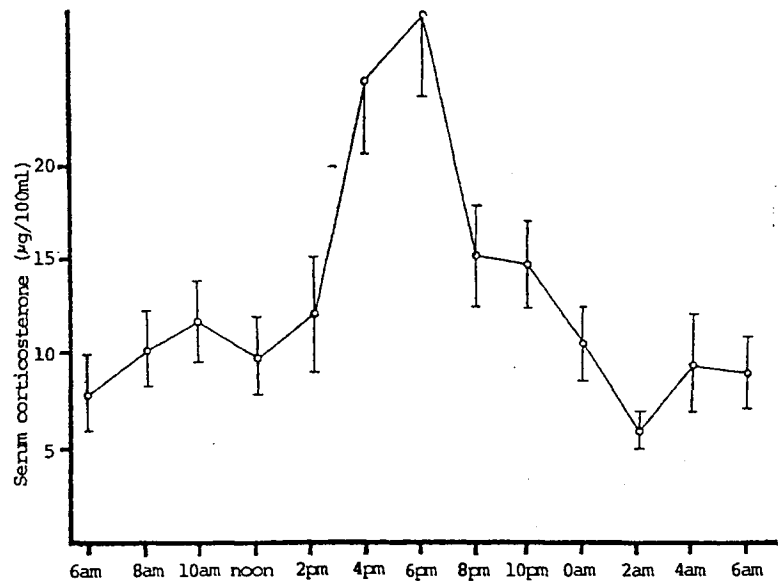


Figure 1.4.3.i (continued). Circadian rhythm of plasma corticosterone secretion in rats (B) and mice (C) – taken from Shimuzu *et al*; 1983.

(B)



(C)



iii) Neurotransmitters - Most of the putative neurotransmitters have been identified in the median eminence of the hypothalamus however, the specific effects of any given neurotransmitter on CRF/ACTH release remain controversial. Current consensus supports the idea of a facilitatory role for NA in the regulation of corticosteroid secretion (Trestman *et al*, 1993). Activation of the HPA response to rising corticosteroid concentrations may be driven by catecholamine-producing pathways that project directly onto CRF neurones in the PVN. These effects appear to be mediated by PVN α-adrenoceptors as clonidine and phenylephrine (α-adrenoceptor agonists) are reported to increase rat plasma corticosterone when injected directly into the PVN

(Kovacs *et al*, 1993). The role of β-adrenoceptors is not clear however, the β-adrenergic agonist, isoproterenol, has been reported to increase corticosterone concentrations and propanolol has been found to block stress-induced ACTH release in one investigation (Saphier & Feldman, 1989). Maccari *et al* (1992) also found NA systems to influence corticosteroid receptors in a region specific manner; MR in hypothalamus and amygdala were reduced, whereas hypothalamic GR were increased following NA lesions using 6-OHDA in ADX rats.

The role of 5-HT in HPA regulation has been extensively debated, with most studies agreeing on an excitatory action of 5-HT on ACTH and corticosteroid secretion (Fuller, 1981; Bruni *et al*, 1982; Fuller, 1992). 5-HT involvement in HPA feedback control is supported by observations that many HPA feedback sites express numerous 5-HT receptors and serotonergic projections to limbic and HPA control sites. 5-HT is also thought to be important in the control of the diurnal cycle of ACTH secretion (Stokes & Sikes, 1987). ACTH and corticosterone release is found to be stimulated by administration of 5-HT precursors (5-HTP, L-tryptophan) and releasing agents (d,l-fenfluramine; Dinan, 1996a; 1996b). There is an abundance of data suggesting a role for 5-HT_{1A} receptors in the regulation of corticosteroid secretion; the 5-HT_{1A} receptor agonist ipsapirone, induces an increase in ACTH and cortisol - this response being blocked by metergoline, a $5\text{-HT}_{1/2}$ antagonist (Welch *et al*, 1993; Dinan, 1996a; Meijer *et al*, 1998). 5-HT_2 receptors are also thought to be involved in the control of HPA activity (Mitchell *et al*, 1990). Lesions of 5-HT neurones, chronic 5-HT depletion and inhibition of 5-HT synthesis are reported to decrease hippocampal GR binding sites and mRNA (Seckl *et al*, 1990, 1992) and increase MR binding sites and mRNA expression (Semont *et al*, 1999). 5-HT also elevates GR, but not MR in cultured hippocampal cells, possibly via an interaction with a 5-HT_2 receptor (Mitchell *et al*, 1990).

Dopaminergic effects on HPA activity are also unclear, with investigations suggesting both stimulatory and inhibitory roles in the control of corticosteroid secretion (Casolini *et al*, 1993; Puri *et al*, 1994). The administration of corticosteroids and CRF is reported to increase vulnerability to the acquisition of an amphetamine self-administration task (Piazza *et al*, 1996) implicating the HPA axis with the modulation of reward systems which could be important in relation to anhedonic symptoms seen in depression.

Other neurotransmitters have also been implicated in HPA regulation. Cholinergic agonists such as carbachol and oxytremorine, have been demonstrated to stimulate HPA axis activity in rats, with this effect being blocked by atropine. Physostigmine, the cholinesterase inhibitor, has been found to increase corticosteroid secretion in rats and humans (Janowsky *et al*, 1972). These data suggest a stimulatory role of acetylcholine on HPA activity. Several studies support a stimulatory role for histamine on HPA activity. It has been suggested however, that the excitatory effects of histamine on secretion of corticosterone and ACTH may be due to peripheral rather than central mechanisms (Calagero *et al*, 1986; 1988b). GABAergic mechanisms are found to exert inhibitory effects on corticosteroid and ACTH release since the GABA antagonists, picrotoxin and bicuculline both stimulate ACTH release (Calagero *et al*, 1988a). Regions associated with inhibitory pathways are reported to contain substantial populations of GABAergic neurones (containing GABA), which are known to inhibit ACTH and corticosterone secretion. The presence of GABA-immunoreactive terminals on PVN neurones has also been demonstrated, implying a direct interaction between GABA and the PVN.

It appears that HPA activity is controlled by a variety of neurotransmitter pathways, a number of which are involved in CRF secretion and terminate on hypophysiotrophic

CRF-synthesising neurones in the PVN. One recent study has suggested that NMDA, GABA-$_A$ and β-adrenoceptors are involved in the down-regulation of hippocampal GR mRNA following stress demonstrating the complexity of interactions involved (Tritos *et al*, 1999). Neurotransmitter control of the HPA axis is complex and the data pertaining to roles of specific neurotransmitters in HPA regulation still requires some clarifying. The bed nucleus of the stria terminalis (BNST) is also thought to communicate additional HPA excitatory information through its links with the amygdala and hippocampus and the hypothalamus and brainstem regions. Aminergic and serotonergic input to the HPA axis from the locus coeruleus and raphe nucleus respectively may occur via other indirect pathways as these regions are reported to have limited innervations of hypothalamic areas. Another recent theory postulates that the changes in monoaminergic pathways observed in depression may occur as a direct result of a hyperactivity of the HPA axis (Dinan, 1994).

iv) Stress - Stress is the primary stimulus of HPA activity. Under stressful conditions, CRF, ACTH and corticosteroid production is increased with the concentrations of corticosteroids produced generally reflecting levels of stress. Very high concentrations of corticosteroids may be secreted and if the response is not adequately regulated at primary (adrenal), secondary (pituitary) or tertiary (hypothalamic) levels, then physiological and psychological problems may ensue (e.g. Addison's disease, Cushing's disease, hypoadrenalism).

The HPA axis responds to stress with increased activity at any time during the circadian cycle of corticosteroid secretion. However, magnitudes of stress responses (ACTH and corticosteroid measurements) are reported to be higher during the nadir of the diurnal cycle than those to the same stress at the peak of the rhythm (Dallman *et al*,

1992). Studies by Bradbury *et al* (1991) have demonstrated that this effect is not due to concentrations of circulating corticosterone, as there is similar stress responsiveness in adrenalectomised (ADX) animals thereby suggesting that this rhythm may have a neural basis.

Initiation of the HPA stress response could be attributed to the excitatory effects of the numerous neurotransmitters (especially NA) and neuropeptides on HPA activity. The process of maintaining corticosteroid secretion within tolerable limits appears to be accomplished by multiple pathways. Corticosteroid negative feedback mainly acts at the PVN neurone itself as demonstrated by the downregulation of CRF mRNA, decreased ACTH secretion and inhibition of PVN neurones following direct injections of corticosteroids into the PVN and also the presence of GR in hypophysiotrophioc PVN neurones (Herman, 1997). Inhibitory influences of corticosteroids are exerted primarily at the hippocampus and multiple levels of the HPA axis (i.e. the pituitary and adrenals). Sapolsky *et al* (1984b) have also reported enhanced restraint-induced increases in plasma corticosteroids following kainate lesions of the hippocampus in which at least 50% of hippocampal neurones were destroyed. This again suggests that the hippocampus is an important site of HPA axis feedback and plays a large role in termination of the stress response. However, the observation that inhibition of ACTH release occurs in the absence of a feedback signal suggests that there may be neuronal pathways involved in the regulation of HPA stress responses in addition to corticosteroid feedback. Lesion studies have also shown that several extrahypothalamic structures such as the locus coeruleus, medial or cortical amygdaloid nuclei, (stimulation of which is found to elicit corticosterone release), lateral septum, prefrontal cortex and hippocampus extend neurotransmitter pathways that project directly onto CRF neurones in the PVN.

The control of stress responses by the HPA system is complex and it appears that stress-induced regulatory feedback circuits may be stressor-specific (Jacobsen *et al*, 1991; Herman, 1997). Lesions of the limbic system are demonstrated to affect HPA responses to restraint, novel environments and fear conditioning (all processive stressors), none of which pose an immediate threat to the animal's physiology. HPA activation by hypoxia, respiratory, cardiovascular and immune stimuli (systemic stressors) are all unaffected by limbic system lesions. This has led to the proposal that there may be two generalised stress pathways within which there are distinct interactions activated by various stressors thus allowing a wide range of information processing mechanisms (Herman, 1997).

During chronic stress, a number of alterations take place within the HPA axis and also in extrahypothalamic structures which are involved in HPA regulation, with definite disruption of corticosteroid inhibitory feedback observed in chronically stressed humans and rats. This will be further discussed in the following section.

1.4.4. The HPA axis and chronic stress

In the 1930's, Selye's "General Adaptation Syndrome" proposed a generalised stress response, biologically effected principally by the corticotrophin-releasing factor (CRF) and locus coeruleus-noradrenaline (LC-NA) systems which became a valuable model linking stress with disease. Following activation of the sympathetic LC-NA system, NA is released resulting in enhanced arousal, cognition, vigilance, concentration and increased anxiety. Activation of the CRF system subsequently leads to increased heart rate, blood glucose and blood pressure and intraventricular injections of CRF are also reported to potently exaggerate behavioural responses, enhancing arousal and promoting cautious restraint whilst concurrently inhibiting vegetative functions such as

feeding and reproduction (Chrousos *et al*, 1992). Administration of the CRF antagonist, α-helical CRF, reverses behaviour reflecting stress/anxiety in rats (Adamec *et al*, 1993b). The LC-NA and CRF systems are closely linked with many potential sites of interaction. It appears that activation of one of these systems will also activate the other in a positive feedback loop. The application of CRF onto LC neurons markedly increases their firing rate, the central administration of a CRF antagonist diminishes the response of the LC to various stimuli while β-adrenergic blockade attenuates the arousal producing effects of centrally administered CRF (Dunn *et al*, 1990; Chrousos *et al*, 1992).

This stress response is initiated when homeostasis is threatened. An important function of corticosteroid release is the counter-regulation of the effectors of the stress response in order to prevent its prolonged and excessive activation. This could occur possibly via the antagonism of CRF and the LC-NA systems directly through CR mediated increase in tyrosine hydroxylase in the LC or indirectly, via effects of CRF on the LC

Recent experiments in humans and animals have demonstrated that the sympathetic adreno-medullary / LC-NA system is preferentially activated in response to acute stress providing the survival aspect of the stress response. The hypothalamic-pituitary-adrenocortical (HPA) axis / CRF system becomes activated following prolonged exposure to the stressor when the subject feels a loss of control. This provides the conservation-withdrawal mode of stress response that is associated with helplessness and depression (Henry, 1993). In larger doses the central administration of CRF produces anxiogenic-like effects including hyper-responsiveness and freezing on exposure to sensory stimuli, decreased exploration in novel environments and enhanced fear-conditioned responses to aversive stimuli (de Boer *et al*, 1990; Dunn *et al*, 1990).

The neuroendocrinological response to chronic stress is achieved at several levels of the HPA axis. Hypersecretion of corticosteroid hormones (corticosterone in the rat and cortisol in humans) constitutes the central drive of the HPA stress response. Consistent increases in corticosteroid secretion have been observed in both animals and humans following exposure to stress (Sapolsky *et al*, 1985; Linkowski *et al*, 1985). The expression of CRF/AVP and mRNA (especially in parvocellular cells of the PVN), is also found to be higher in stress and depression (Nemeroff *et al*, 1984; Raadsheer *et al*, 1994; Ur *et al*, 1995; Schulkin *et al*, 1998). Chronic stress also appears to result in defective negative feedback in many of the stress paradigms investigated - thought to be due to a down-regulation of CR predominantly in the hippocampus (Young *et al*, 1990; Stec *et al*, 1994). Hypertrophy of the adrenal glands is also commonly reported following most forms of chronic stress (Herman *et al*, 1995).

The acute stress response is normally controlled by negative feedback at various levels of the HPA axis (see section 1.4.1.i). In the presence of chronic stress, the increased secretion of corticosteroid hormones is maintained despite negative feedback control mechanisms, resulting in many damaging symptoms. While response to stress is a necessary survival mechanism, prolonged stress can have severe repercussions leading to enhanced neuronal cell death and resulting in a variety of cognitive defects (Chrousos *et al*, 1992 - see Table 1.4.4.i; Sapolsky, 1996). Therefore it appears that a balanced and well-regulated HPA system may be very important for both physiological and psychological stability. This is important as the syndrome of melancholic depression also seems to represent a dysregulated generalised stress response (Table 1.4.4.i.) – this is discussed further in the next section.

Table 1.4.4.i. Parallels between Selye's "General Adaptation Syndrome" and symptoms of depression. Modified from Gold *et al* (1988).

OBSERVED ALTERATIONS	ACUTE STRESS	DEPRESSION
Redirection of behaviour by the central nervous system	• Acute facilitation of adaptive neural pathways • Arousal, alertness • Increased vigilance, focused attention • Aggressiveness when appropriate • Acute inhibition of non-adaptive pathways • Decreased eating • Decreased libido and sexual behaviour • Appropriate caution or restraint	• Chronic maladaptive facilitation of neural pathways • Dysphoric hyper-arousal and anxiety • Hyper-vigilance, constricted focus, obsessionalism • Assertiveness inappropriately restrained by anxiety • Maladaptive inhibition of neural pathways • Decreased eating • Decreased libido and sexual behaviour • Excessive caution, regardless of context
Redirection of energy in the periphery	• Oxygen and nutrients to the stressed body site • Increased blood pressure, heart, and respiratory rates • Increased gluconeogenesis • Increased lipolysis • Inhibition of programs for growth and reproduction • Acute glucocorticoid-mediated counter-regulatory responses (containment) • Restraint of corticotropin-releasing-hormone system and the pituitary-adrenal axis • Restraint of the norepinephrine-locus coeruleus system • Restraint of the expected immuno-logic or inflammatory response	• Oxygen and nutrients to the central nervous system • Increased blood pressure, heart, and respiratory rates • Increased gluconeogenesis • Increased lipolysis • Inhibitions of programs for growth and reproductions • Chronic inadequate or maladaptive counter-regulatory responses (containment) • Inadequate restraint of the corticotropin-releasing- hormone system and the pituitary-adrenal axis • Inadequate restraint of the norepinephrine-locus coeruleus system • Chronic immunosuppression

1.4.5. The HPA axis and chronic stress/depression

The acute behavioural and physiological alterations that occur during the general adaptive syndrome are very similar to the chronic changes observed in major depression with many common features appearing to be consequences of the CRF and locus coeruleus-NA systems (Gold *et al*, 1988 – see Table 1.4.4.i). Features such as elevated cortisol secretion and adrenal hypertrophy (Rubin *et al*, 1995; Nemeroff *et al*, 1992) are commonly observed in depressed subjects as well as in chronically stressed animals (Marti *et al*, 1994; Herman *et al*, 1995). This implies that the symptoms of major depression may originate from acute stress responses that are maladapted and have eluded the normal regulatory controls.

Maintained hypersecretion of cortisol has consistently been demonstrated in the majority (>50%) of patients with depression, with increased basal concentrations of cortisol being found in urine, plasma and CSF (Carroll *et al*, 1976). This is thought to result from defective negative feedback which is frequently measured using the DST (see section 1.4.3.i), a test of neuroendocrine function in which depressed patients are found to have an abnormal early escape of plasma cortisol during a 24 hour overnight dexamethasone suppression test (Carroll *et al*, 1981; Heuser *et al*, 1994). The circadian rhythm of cortisol secretion is disrupted in depressed patients showing increased magnitudes, frequency and duration of secretory episodes (Linkowski *et al*, 1987). There appears to be some conflict regarding ACTH concentrations in depressed patients with some studies showing elevated ACTH in depression and others reporting no differences between depressed and control subjects (Yerevanian *et al*, 1983; Linkowski *et al*, 1985).

There is also some evidence of increased CRF neurone activation leading to increased CSF concentrations of CRF in depressed patients (Nemeroff *et al*, 1984) and an increased pool of CRF in the median eminence following repeated stress in rats (Inoue *et al*, 1993). Elevated CRF concentrations are thought to be, at least partly responsible for the hyperactivity of the HPA axis seen in depression and mediation of some behavioural symptoms involving sleep and appetite disturbances and changes in libido and psychomotor activity (Ur *et al*, 1995; Mitchell, 1998; Arborelius *et al*, 1999). One study has reported a reduction in the number of CRF receptors in the frontal cortex of suicide victims (Nemeroff *et al*, 1986). Increased CRF receptors have also been demonstrated in amygdaloid and hypothalamic regions of the rat brain following repeated stress (Haugher *et al*, 1993; Garcia-Garcia *et al*, 1998; Mansi *et al*, 1998). Increased numbers of CRF expressing and CRF/AVP co-expressing neurones have also been observed in the PVN of depressed patients (Raadsheer *et al*, 1994).

Disturbances in corticosterone and ACTH secretion have also been reported in some animal models of depression (Cairncross *et al*, 1977; Marcilhac *et al*, 1997) and in numerous studies involving chronically stressed animals (Sapolsky *et al*, 1984; Dallman *et al*, 1987; Hashimoto *et al*, 1988; Lachuer *et al*, 1994). However, several investigations have not detected any increases in corticosterone and ACTH in stressed animals thus suggesting that these responses may be stressor specific (Hashimoto *et al*, 1988; Azpiroz *et al*, 1999). Increased CRF concentrations and expression have been demonstrated in the median eminence and hypothalamus following stress with reduced CRF receptor binding and expression after stress (Inoue *et al*, 1993; Plotsky *et al*, 1993; Smith *et al*, 1998).

As mentioned earlier, defective negative feedback control of the HPA axis (displayed by DST non-suppression) is commonly observed in depression. The ACTH/β-endorphin test is also impaired in depression providing evidence for defective negative fast feedback control of the HPA axis (Young *et al*, 1991). Dexamethasone resistance has also been reported in chronically stressed animals (Brooke *et al*, 1994a; Barden *et al*, 1997).

Impaired negative feedback of the HPA axis, as seen in depression, has often been attributed to a down-regulation of brain CR. This is particularly important with regards to this thesis and several investigations have generated data consistent with the hypothesis that an altered CR function or capacity underlies the exaggerated HPA response in depression. Using the combined dexamethasone/CRF test, Modell *et al* (1997) demonstrated that decreased feedback control of the HPA axis in depressed patients was likely to be due to a failure of GR-mediated feedback action and that CR function is reduced in depressed patients. Investigations of CR numbers in humans have been inconsistent with some studies showing reductions in CR in depressed patients and others showing no differences between control and depressed subjects (using whole cell and cytosolic assays in mononuclear cells, lymphocytes and cultured skin fibroblasts; reviewed in Pariante *et al*, 1995). Post-mortem studies in suicide victims have also shown an altered ratio of MR/GR in the CA3 subfield of the hippocampus (Lopez *et al*, 1998). Investigations involving the measurement of CR in peripheral tissues (lymphoid tissue, mono- and polynuclear leukocytes) have yielded inconsistent data with some studies showing reductions in MR following high cortisol (Armanini *et al*, 1994) and others showing no alterations in CR binding (Wassef *et al*, 1992). Investigations in rats by Spencer *et al* (1991), which have demonstrated that the

regulation of GR in neuronal and lymphoid tissues is similar, do not appear to clarify these data.

Several animal studies have demonstrated selective decreases in GR following chronic stress (Sapolsky *et al*, 1984a; Meaney *et al*, 1989) and some investigations have demonstrated increased corticosterone secretion following the depletion of GR or the destruction of glucocorticoid-sensitive hippocampal neurones (Sapolsky *et al*, 1984b, 1985). Brooke *et al* (1994) has also demonstrated that dexamethasone resistant primates with a history of social instability had significantly fewer available hippocampal GR binding sites than dexamethasone responsive animals. Sustained elevations of circulating corticosterone by exogenous corticosterone administration have also resulted in reduced CR numbers in several studies (Sapolsky *et al*, 1985; Spencer *et al*, 1991). Chronic, stressful stimuli have been shown to result in decreased GR mRNA levels in the hippocampus (Herman *et al*, 1995; Gomez *et al*, 1996; Kitraki *et al*, 1999).

It is quite possible that the primary site of action of stressful influences may be the GR as it is known that, in transgenic animals with disrupted GR function, a secondary HPA activation develops along with various feeding, cognitive and behavioural deficits. Transgenic mice with impaired GR function display elevated corticosterone and ACTH concentrations, significant dexamethasone non-suppression, reduced GR binding and hypertrophic adrenal glands (Pepin *et al*, 1992c; Stec *et al*, 1994; Barden *et al*, 1997) as compared to normal animals.

Chronically elevated corticosteroid concentrations result in numerous defects leading to neurodegeneration or suppressed neurogenesis in the hippocampus via altered

hippocampal transmission. Pyramidal neurones in the CA3 region of the hippocampus appear to be particularly susceptible with some defects also reported in the CA1 and dentate gyrus (Beck *et al* ,1994; Magarinos *et al*, 1997). An imbalanced CA3/CA1 network may favour excitatory over inhibitory signals and lead to increased Ca^{2+} influx, which along with elevated GR occupation, is a prominent feature of corticosteroid hypersecretion (de Kloet, 1998). Alterations in glutamatergic transmission are also observed following high corticosteroid concentrations (GR activation has been demonstrated with numerous other factors involved in synaptic transmission). Hippocampal brain-derived neurotrophic factor (BDNF) and tyrosine kinase B expression is reduced following exposure to corticosteroids. The activation of GR also alters synthesis of nerve growth factor, transforming growth factor and glycoprotein cell adhesion molecules (de Kloet, 1998). High corticosteroid concentrations may therefore exert their damaging effects via these GR-related mechanisms.

A key role has also recently been suggested for CRF receptors in mediation of stress responses following consistent demonstrations of alterations involving CRH/receptors (as summarised earlier – reviewed by Mitchell, 1998). Gene targeting experiments have also shown that mice with disrupted CRF_1 receptor genes exhibit impaired stress responses, reduced anxiety and defective HPA axis development and activity (Smith *et al*, 1998; Timpl *et al*, 1998). The relationship between CRF receptors and CR is likely to be complicated as both are involved in feedback regulation of the HPA axis and may impact at several sites. Lechner *et al* (1999) have shown GR immunoreactivity in the bed nucleus of the stria terminalis (BNST) and Barrington's nucleus, both CRF releasing afferents to the LC and have suggested that alterations in corticosteroid concentrations or GR functions may affect the activity of these CRF systems and

ultimately LC function. These consistently observed abnormalities in HPA axis function have led to the proposal that these may be the primary mediators of monoamine and immunological disturbances in stress and depression. Some evidence has accumulated to support the hypothesis that activation of the HPA by chronic stress may, via high levels of GR on central neurones, produce changes in central monoamines (de Kloet *et al*, 1986; 1987; Dinan, 1994).

NA stimulated cAMP formation in the cortex and hippocampus is found to be suppressed following high corticosteroid concentrations (de Kloet *et al*, 1986). α_2-Adrenoceptors in the PVN are significantly down-regulated following ADX, an effect which is prevented by corticosterone administration (Dinan, 1995). β_1-Adrenergic receptors are also down-regulated by corticosteroids with a significant up-regulation of β_2 adrenoceptors in rat C-6 glioma cell cultures (Kiely *et al*, 1994). Patients displaying dexamethasone non-suppression were also found to show a blunting of the NA mediated growth hormone response (Dinan, 1996a; 1996b).

5-HT turnover is significantly decreased in the hypothalamus, hippocampus and raphe nucleus (all target sites for corticosteroid feedback) of ADX rats; these being normalised by corticosterone substitution (de Kloet *et al*, 1991). 5- HT_{1A} receptors are also found to be regulated by corticosteroids (Chalmers *et al*, 1993) and it is possible that 5-HT receptor subsensitivity may be partly induced by high corticosteroid concentrations as demonstrated by a blunted fenfluramine stimulated prolactin response. Other neurotransmitter abnormalities have not been intensely investigated however, ACh and GABA may also be involved and regulated to some degree by HPA disturbances.

It is clear that primary reductions of CR result in elevated corticosteroids, which may subsequently alter corticosteroid-sensitive neurotransmitter gene expression which in turn, results in neurotransmitter imbalance. Corticosteroid receptors appear therefore to play an important role in the pathogenesis of depression and stress-related disorders. This idea is further supported by observations that MR/GR can be manipulated by the administration of various antidepressant compounds.

1.4.6. The HPA axis and antidepressants

Initial acute actions of most antidepressant drugs involve the blocking of neurotransmitter reuptake or inhibition of neurotransmitter degradation. In addition to these activities, many antidepressant drugs are found to exert effects on HPA function. The primarily NA blocking drugs, desipramine (DMI) and oxaprotiline (S+ enatiomer) are found to activate the HPA axis, possibly via effects at α_1 adrenoceptors. Fluoxetine, a 5-HT reuptake inhibitor, is found to elevate plasma CRF concentrations in rats (Holsboer & Barden, 1996).

Chronic administration of various antidepressants has been demonstrated to normalise defective HPA activity as assessed by corticosteroid hypersecretion in depressed patients (Barden, 1995; Holsboer & Barden, 1996). Several animal studies also support this finding. Shimoda *et al* (1988) found that chronic administration of imipramine, clomipramine and desipramine suppressed plasma corticosterone release in rats. Reul *et al* (1993; 1994) demonstrated that control rats exposed to novel environment stress secreted more ACTH and corticosterone than rats receiving amitriptyline or moclobemide prior to exposure. Chronic administration of tianeptine, a novel antidepressant, attenuated HPA activation in stressed animals by inducing a decrease in plasma ACTH and corticosterone levels, along with significant reductions in CRF

concentrations in the hypothalamus (Delbende *et al*, 1994). Long term administration of imipramine is also reported to reduce CRF mRNA levels in the PVN (Brady *et al*, 1991). In parallel with these findings, CRF binding capacity in the anterior pituitary is reduced following long term antidepressant administration (Reul *et al*, 1994).

Administration of antidepressants may attenuate HPA hyperactivity by normalising the defective negative feedback observed in depression. Many patients who are non-suppressors in the DST or dexamethasone-CRF test display dexamethasone suppression after being given long-term antidepressant treatment (Holsboer & Barden 1996). Concentrations of CRF in CSF are also reduced following antidepressant administration (Holsboer & Barden, 1996). These results suggest that the long term administration of antidepressants may suppress the HPA system thereby raising the possibility that lowered HPA activity and a clinical response are causally related. Antidepressant effects on some neuroendocrine parameters can be seen within minutes of their administration. Torres *et al* (1998) showed that fluoxetine induced CRF and CRF_1 receptor mRNA expression 30 minutes after administration. However the clinical effects of antidepressants are not usually observed for 2 weeks or more therefore suggesting that the acute effects of antidepressant administration such as inhibition of neurotransmitter reuptake may constitute the start of a cascade resulting in the lowered HPA activity. The time course of CR changes following antidepressant treatment in many previous animal studies is found to be similar to that of onset of therapeutic changes (Reul *et al*, 1993; 1994) indicating that regulation of CR may form at least partly, the mechanism by which antidepressant compounds exert their clinical effects.

A number of studies have investigated the effect of repeated administration of various antidepressant drugs on CR binding and expression. Kitayama *et al* (1988) found

increased brain GR immunoreactivity after chronic administration of imipramine. Chronic (over two weeks) exposure to various antidepressants (amitriptyline, moclobemide, imipramine, maprotiline) has been reported to increase MR and GR binding activity, mostly in rat hippocampal and hypothalamic regions (Reul *et al*, 1993; Reul *et al*, 1994; Budziszewska *et al*, 1994a; 1994b; Okugawa *et al*, 1999). The expression of GR and MR mRNA in rat brain and cell cultures is also found to be enhanced following long-term administration of the antidepressants imipramine, amitriptyline, desipramine, citalopram and maprotiline (Pepin *et al*, 1989; Peiffer *et al*, 1991; Seckl & Fink, 1992; Pepin *et al*, 1992a; Barden, 1996 and Pariante *et al*, 1997). GR gene promoter activity in cell cultures is also stimulated by antidepressants (Pepin *et al*, 1992a, Okugawa *et al*, 1999) and a number of different antidepressants are found to modulate this receptor *in vivo* (Peiffer *et al*, 1991).

Barden *et al* (1995) suggested that antidepressants may aid in the maintenance of GR function in NA and 5-HT containing cell groups, an interesting hypothesis as the hippocampus, intricately involved in stress and depression related symptoms, receives ample NA and 5-HT innervations. A possible mechanism for this may involve the antidepressant-induced modulation of GR gene expression, which as a primary effect of antidepressant action would result in decreased HPA activity including a reduction in CRF gene expression. Secondary effects would therefore occur as a result of reduced corticosteroid concentrations and be exerted on corticosteroid-sensitive processes, such as neurotransmitter biosynthesis, leading to more balanced neurotransmitter processes. (Figure 1.4.6.i).

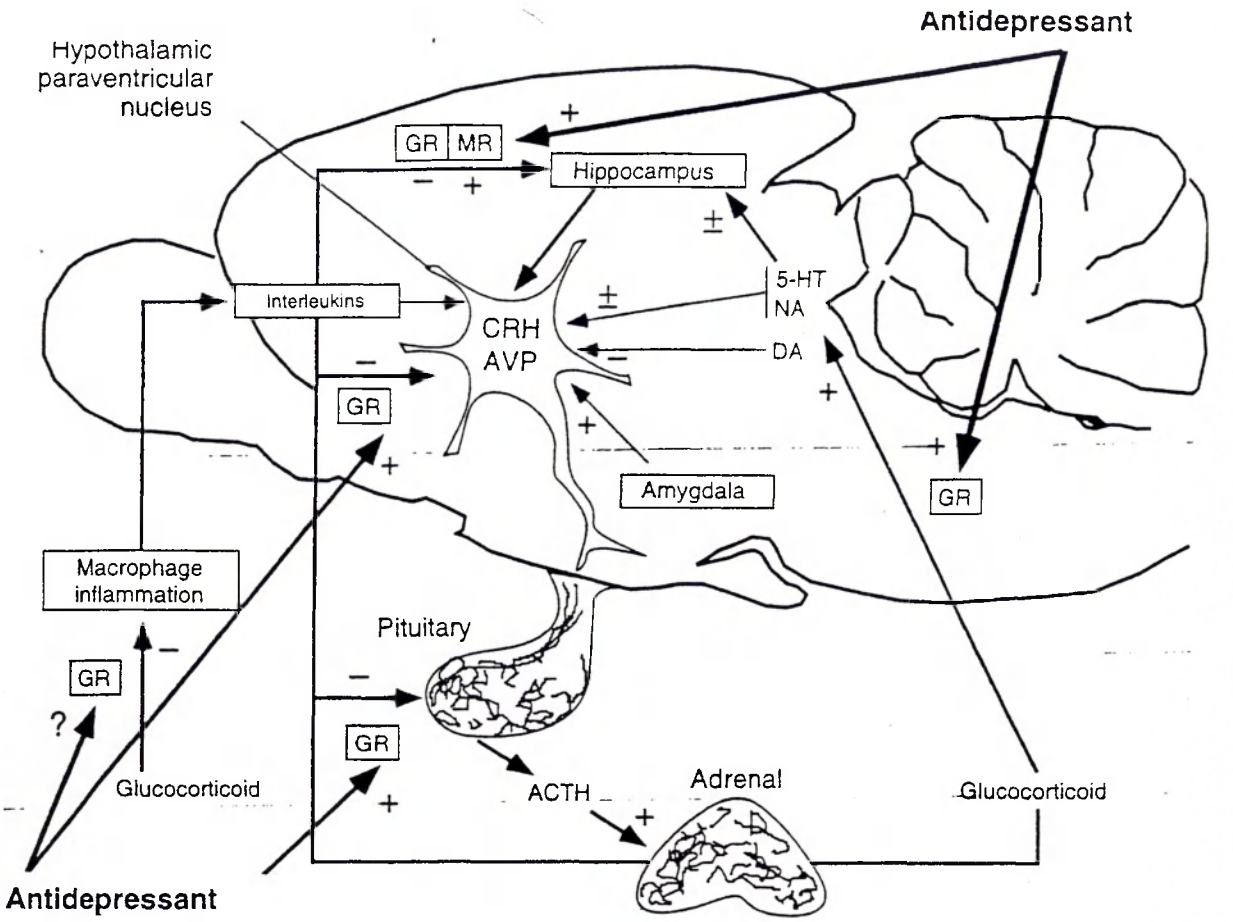


Figure 1.4.6.1. Antidepressant induced increases in glucocorticoid receptor (GR) and mineralocorticoid receptor (MR) gene expression suggest a novel mechanism of action of these drugs on the hypothalamic-pituitary-adrenocortical (HPA) system. This figure shows stimulatory (+) and inhibitory (-) actions of neural inputs to brain regions involved in HPA regulation and to sites of corticosteroid retroinhibition. Arrows indicate the sites at which antidepressants can have stimulatory actions on GR and MR. Taken from Barden *et al* (1995).

The recent development of transgenic mice with primary GR defects has been extremely useful in elucidating HPA and related antidepressant mechanisms in stress and depression (Pepin *et al*, 1992b; Barden, 1996). Transgenic mice display higher ACTH and corticosterone levels, decreased GR mRNA and numerous behavioural deficits (Pepin *et al*, 1992b; 1992c; Stec *et al*, 1994) which are normalised following antidepressant administration and appear to be a valid model for the neuroendocrinological disturbances seen in depression. However, the evidence is not entirely consistent, due to difficulties in attempting to establish clinically relevant effects in laboratory animals.

Many aspects of neuroendocrine dysfunction following stress and anxiety can be investigated via animal behaviour and biochemical procedures. One of the most problematic aspects of researching depressive syndromes however, is the lack of appropriate models on which to test various hypotheses – many of which are extended theories of drug action and as such, do not provide any solid evidence. Also, much of the clinical evidence gathered is from tissues taken from suicide victims and may be subject to artefacts resulting from death. As a result, investigations involving the simulation of depressive symptoms in animals have become increasingly important in studying the psychobiology of depression in humans.

1.5. Animal models of depression

Recently, there has been a major expansion in the number of behavioural paradigms being proposed as animal models of depression. This situation has evolved through two main observations. Firstly, that there may be environmental precipitants to depression – which has subsequently led to the use of animal models to determine the relationship between anxiety, stress and depression. Many of the animal models being used to study depression today include a strong stress component, e.g. learned helplessness (King *et al*, 1993), behavioural despair (Porsolt *et al*, 1977; 1978; Hansen *et al*, 1997), chronic mild stress (Willner *et al*, 1992). Secondly, the potentially significant effects of chronic antidepressant administration on various biochemical parameters have also resulted in an increased number of studies correlating these with changes in behaviour that may follow a similar course. The greatest contribution of animal models at present lies in the investigation of the mechanism of action of antidepressant drugs. The chronic administration of antidepressants causes a variety of changes in the functioning of neuronal systems (as discussed earlier) and animal models can be used to establish the significance of these changes.

Several symptoms of depression, excluding subjective feelings such as dysphoric mood, feelings of guilt and thoughts of suicide, can be translated into measurable behaviours. Psychomotor changes, agitation or retardation are well documented symptoms of stress and depression and can be measured with relative ease in animals. Loss of motivation, or insensitivity to rewards (anhedonia) is a major feature of endogenous depression that has been successfully simulated in a number of behavioural paradigms. These are central features of depression which have been simulated and measured in a number of animal models of depression and which form the basis of the animal model investigations in this thesis. Other features that have been studied include learning deficits, decreased persistence, decreased sexual activity and disturbed sleep or food intake.

The validity of the model is an important consideration when attempting to simulate human syndromes in a different species (Willner, 1991a; 1991b), this generally being assessed on the following groups of relatively independent criteria. Face validity refers to the degree of symptomatic resemblance between the model and the clinical condition. Predictive validity is primarily concerned with correspondence between drug actions in the model and in the disorder and finally, construct validity addresses the theoretical rationale of the model. Very few animal models display strong validity in all three of theses areas and the presence of a number of other features in the model are also desirable such as the modelling of an appropriate core symptom or the demonstration of a number of core features of the disorder. A realistic method of inducing symptoms and responsiveness to antidepressant treatment, preferably over a prolonged time course is also desirable.

Animal models have been used during the course of this thesis to investigate the effects of various antidepressants on a number of behavioural parameters. In the context of studying the mechanisms of action of antidepressant drugs, the use of animal models in this thesis is required in order to investigate the changes, if any, in HPA function in these animal simulations of depressive symptoms. The large number of proposed animal models of depression have been the subject of a number of reviews (Jesberger & Richardson, 1985; Willner, 1991a; 1991b, Kelly *et al*, 1995) however for the purposes of this thesis, we will consider the relevance of two of these models in detail.

The olfactory bulbectomised rat model of depression makes use of surgical lesioning to induce a depression-like syndrome in rats. This model exhibits a number of the characteristics required of an appropriate model of depression and has received much attention as a model with which to screen compounds for potential antidepressant activity (Jesberger & Richardson, 1985; Van Riezen & Leonard, 1990). The bulbectomised rat, which possesses reasonable face and predictive validity displays many of the behavioural, neurochemical and immunological changes associated with depressed patients (see Table 1.6.1.i). There is an abundance of experimental evidence to suggest that the olfactory bulbectomised rat may fulfil many of the criteria required of an animal model of depression (Chapter 4).

The induction of stress responses in animals by various means has provided a basis for many models of anxiety and depression and many behavioural paradigms being proposed as animal models of depression involve the long term exposure to one or more stress inducing stimuli. Recent investigations have involved the exposure of animals to a predator or its odour and have shown that this exposure can result in numerous behavioural and neurochemical stress responses in various species (File,

1996). The proposed predator stress "model" is a relatively recent behavioural paradigm and possesses strong construct validity. There is some evidence that this paradigm may also display some face and predictive validity as a model of stress/depression however, given that this paradigm has not yet been established, much work is required to clarify its status as a model for depression (Chapters 5 and 6).

Table 1.6.1.i. Similarities between depressed patients and rats subjected to olfactory bulbectomy and predator stress
(partly adapted from Jesberger & Richardson,1988).

Depressed patient	Olfactory bulbectomised rat	Predator stress
Changes in appetite and weight loss	Abnormal daily eating pattern and reduced weight gain. Impaired food-motivated behaviour	Reduced growth. Reduced appetite.
Loss of interest in sexual activity	Abnormal lordosis and other sexual behaviour	Not yet determined.
Disturbed sleep patterns	Abnormal sleep patterns	Not yet determined.
Cognitive deficits	Impairments in avoidance and spatial tasks and food motivated behaviours	Impaired risk assessment behaviour.
Agitated and irritable	Hyperactive, reactive, increased aggression and nuricide	Increased proxemic avoidance behaviour. Altered activity patterns.
Inability to experience pleasure	Anhedonic responses - Deficits in behavioural tasks involving positive reinforcement	Not yet determined.
Altered functioning of NA, 5-HT, GABAergic, cholinergic and other neurotransmitter systems	Neurochemical disturbances including abnormal NA, 5-HT, GABAergic and cholinergic systems.	Altered functioning of GABA, benzodiazepine and 5-HT systems.
Hyperactivity of the HPA-axis. Increased plasma cortisol levels.	HPA-axis disturbances evident. Increased plasma corticosterone levels reported.	HPA axis activity increased. Increased corticosterone, ACTH and CRH concentrations reported.
Immune system alterations including reduced lymphocyte and neutrophil phagocytosis. Increased neutrophils and acute phase proteins	Immunological abnormalities include decreased lymphocyte and neutrophil phagocytosis. Increased neutrophils and acute phase proteins	Not yet determined.
Respond to chronic antidepressant therapy	Respond to chronic, not acute, antidepressant administration	Reduction of stress-induced behaviours by chronic antidepressant administration.

1.6. Objectives of this thesis

Several different types of antidepressants have been demonstrated to increase CR binding and mRNA in various regions of the rat brain over a period similar to the time course in which therapeutic effects are observed in depressed. For this reason, it has been suggested that the up-regulation of CR, thus normalising defective HPA activity, may be a mechanism common to the different classes of compounds used in the therapy of depression.

Many studies have investigated CR alterations following acute or repeated stress procedures and/or antidepressant administration in adrenalectomised, 'naïve' animals yet little attention has been given to any possible changes in CR in intact animals and/or in established and proposed animal models of depression. Given the features of the OB rat model of depression (chapter 4), it could be expected that a dysfunctional HPA axis as reflected by defective negative feedback resulting from stress-induced CR down-regulation would be observed. As yet, no CR measurements have been performed in the OB rat. The predator stress paradigm has not been studied or developed to the extent that the OB rat has, therefore requires substantial behavioural and endocrinological investigations (chapter 5 and 6).

The work presented in this thesis is primarily concerned with the monitoring of plasma corticosterone and corticosteroid receptor alterations under various stress situations and following administration of a variety of antidepressant drugs. CR were quantified using radioligand-binding assays and plasma corticosterone measured using high performance liquid chromatography or radioimmunoassay.

Attempts were also made to compare endocrinological measures of plasma corticosterone and CR with behavioural parameters (locomotor activity and/or anhedonia) as measured in the olfactory bulbectomised rat, and a stress-related paradigm comprised of predator stress exposure. Therefore the objectives of this work were;

i) to investigate the effects of administration of various antidepressants (in rats) on plasma corticosterone secretion and CR binding in various brain regions and peripheral tissues of intact rats.

ii) to examine the effects of olfactory bulbectomy on locomotor activity and brain corticosteroid receptor binding in rats. The effects of administration of various antidepressant drugs on locomotor activity and CR binding were also investigated.

iii) to establish a behavioural paradigm based on predator stress which could be expanded to a potential model for chronic stress/depression.

iv) to determine the effects of predator stress and administration of various antidepressants on locomotor activity and anhedonic measures in mice. Effects of predator stress and antidepressant administration were also investigated on plasma corticosterone and corticosteroid receptor binding in various brain regions.

CHAPTER 2

GENERAL METHODS

2.1. Introduction

This initial experimental chapter describes the basic procedures for performing the corticosteroid receptor (CR) radioligand binding assay used extensively throughout this thesis. The definition of optimal assay conditions and investigation of some established receptor binding criteria are also described. The aim of this chapter is to demonstrate that the specific binding of ^{3}H-dexamethasone to sites in rat brain tissue preparations fulfils the necessary binding criteria and generates reliable data.

2.2. Introduction to radioligand binding studies

Prior to the widespread use of *in vitro* binding assays, the properties of receptors were inferred from the measurement of biological responses. The utilisation of radioligand binding assays to characterise receptors provides an enormous amount of information and also a direct approach to the study of drug-receptor interactions.

2.2.1. Basic criteria for receptor binding studies

Radioligands provide precise probes that allow the specific investigation of the interactions between a drug and its binding site. Usually, ligands that exhibit a high selectivity and affinity for the receptor system being studied are chosen for binding assays.

An essential initial undertaking when performing binding assays is to establish the nature of the site in the tissue preparation to which the radioligand is binding. The hypothesis that a given binding site represents the receptor for the particular radioactive ligand employed for the assay can be substantiated by fulfilling a number of important criteria. Some of these criteria (which are discussed in greater detail in the appropriate sections) include;

1) saturability of the binding of radioligand to the site of interest (see section 2.4).
2) investigations of the pharmacology of binding between the radioligand and binding site via competition by non-radioactive drugs (see section 2.5).
3) the establishment of a linear relationship between tissue concentration and the number of binding sites (see section 2.6).
4) temperature dependence of radioligand binding (see section 2.7).
5) kinetics (association and dissociation) of binding of the radioligand (see section 2.8).

These criteria were experimentally tested and attempts were made to establish i) the site to which the radioligand was binding as a pharmacological or physiological receptor and ii) the optimal conditions for binding of the ligand to the receptor. This was achieved through the combined use of published information and methodology developed in our own laboratory.

2.2.2. Receptor binding assay procedure

Tissue preparations containing the receptor of interest are incubated to equilibrium with one or more concentrations of the appropriate radioligand. Separation of the bound from free ligand is dependent on the equilibrium dissociation constant (K_D) of the radioligand for the binding site. For ligands with K_D values of 10^{-8}M or less, the rapid vacuum filtration method is generally most appropriate whereas for ligands with higher K_D values, separation by centrifugation, column chromatography and precipitation of ligand-receptor complex and adsorption of free ligand techniques are applied (Yamamura, 1990; Bylund, 1992). Measurement of the bound radioactivity trapped on the filter following the separation is by liquid scintillation counting.

The radioactivity bound to the filter is termed the **total binding**. This consists of:

i) **specific receptor binding** - radioligand bound to its binding site on the receptor of interest and;

ii) **non-specific binding** - radioligand bound to other non-receptor components in the tissue preparation (e.g. membrane proteins and lipids, glass fibre filters).

The non-specific component of this binding can be measured by the inclusion of parallel samples of a non-radioactive compound that is known to interact with the receptor system under investigation at a concentration sufficient to totally displace the radioligand from specific binding sites.

The two main types of radioligand experiments 1) saturation experiments and 2) competition experiments are described in detail following a general description of the CR assay (sections 2.4 and 2.5 respectively).

2.2.3. Additional CR binding experiments

Various other preliminary CR binding experiments were carried out to investigate practical considerations relating to future studies. These studies are presented individually in this chapter along with results and analysis of data.

2.3. Corticosteroid receptor binding assay using rat brain tissues

2.3.1. Animal husbandry

Experiments were carried out using male Sprague-Dawley rats (body weight 200 - 250g). Animals were housed 4 per cage (45cm x 25cm x 20cm) and maintained in a temperature (19-22°C) and light (light period 07.00-21.00 hours) controlled room. A standard laboratory diet and drinking water were available *ad libitum.*

2.3.2 Dissection of rat brain regions

All sacrifice and dissection procedures were conducted between 09.00 – 11.00hrs, a time when plasma corticosterone concentrations were low (see Figure 1.4.3.B). Rats were killed by cervical dislocation and decapitation. Trunk blood was collected in plastic Falcon tubes and prepared for serum extraction (as described in section 3.2.4). The brain was rapidly removed from the skull and placed on an iced glass plate prior to blunt dissection procedures. Cortical tissue was used for all experiments in this chapter except those investigating the effect of freezing on the binding parameters of corticosteroid receptors in the hippocampus (section 2.9). Blunt curved iris forceps were used to separate the cortices and layer them to either side of the mid-line. The clearly visible striata were pinched out with the forceps. The hippocampal and amygdala regions were then excised from the brain followed by the cortices and hypothalamus. Brain regions were immediately placed in cryovials, frozen on dry ice and stored at -70°C, or prepared directly for the assay (as required for the experiments). The distribution of corticosteroid receptors in a peripheral tissue, the thymus, was also investigated in some experiments, therefore this organ was also dissected from the body cavity of the animal.

2.3.3 *Radioactive ligand*

^{3}H-Dexamethasone (specific activity 81 – 89 Ci/mmol) was obtained from Amersham International Plc. The ligand was kept at -20°C and diluted to the required concentrations with incubation buffer on the day of the experiment.

2.3.4 *Buffers*

For list of drugs and chemicals (and suppliers), see Appendix 2.3.1. All buffers for the receptor binding assays were made up in distilled water. The required pH at 20°C was achieved by using 6M HCl (hydrochloric acid) for all the buffers. The buffers were kept at 4°C or on ice throughout the experiments.

i) Incubation buffer (pH 7.4):

Component (final assay concentration)

10mM	Tris
1mM	EDTA (disodium salt)
35mM	Sodium molybdate
1mM	Dithiothreitol
10%	Glycerol

ii) Wash buffer (pH 7.4):

10mM	Tris-HCl buffer

2.3.5 *Tissue Preparation*

Cortical tissue was homogenised in 20 volumes (v/w) of ice-cold incubation buffer (pH 7.4) using a motor driven Teflon pestle (Heidolph R2R2050, LabPlant, U.K.) and glass homogeniser (10 slow up/down strokes at 1400rpm). The resulting homogenate was

centrifuged at 105,000g for 60 minutes at 4°C in a Beckman L-70 Ultracentrifuge (Beckman Instruments, Beckman Instruments Inc, U.S.A.) fitted with a 70.1Ti rotor. Tissue supernatant was immediately decanted and stored on ice until required for assay.

2.3.6 CR binding assay incubation procedure (general)

All assays were performed in 10mL borosilicate test tubes (16mm x 16mm x 100mm) which were maintained on ice throughout the assay procedure. The total incubation volume for the assay was 250µL consisting of:

i. 100µL of tissue cytosolic preparation (equivalent to 5mg wet weight of the original tissue)
ii. 100µL of ^{3}H-dexamethasone solution (0.625-20nM final assay concentration)
iii. 50µL displacing compound (at a range of concentrations) or 10% EtOH (the diluent for the displacing compound)

Total binding containing the tissue preparation, ^{3}H-dexamethasone and 10% EtOH was determined in duplicate.

Non-specific binding containing the tissue preparation, ^{3}H-dexamethasone and the displacing agent was determined singly or in duplicate.

^{3}H-dexamethasone standards were prepared, in duplicate, on each experimental day and counted to determine the total assay concentration of radioligand.

The assay was initiated by the addition of tissue supernatant following which the tubes were thoroughly mixed and placed in a refrigerator, at 4°C, for approximately 24 hours (20-26 hours). Tissue supernatant was also stored at -70°C for subsequent protein determination using the method of Lowry (Lowry *et al*, 1951).

2.3.7 Separation of bound and free radioligand

The assay was terminated by rapid filtration under vacuum (600mm Hg) through Whatman glass fibre GF/F filter strips (Whatman International Ltd, U.K.), which had been pre-treated for approximately 24 hours with 0.3% polyethylenimine (PEI), using a Brandel Cell Harvester (Semat Technical Ltd, U.K.). The filters were quickly washed with 16mL of ice-cold 10mM Tris-HCl wash buffer (pH 7.4). This minimised the amount of non-specific binding bound to the glass fibre filters. The procedure of filtration and washing was conducted over approximately 15 seconds.

2.3.8 Determination of radioactivity and scintillation counting

The glass fibre filters were cut out of the strips and placed into individual 10mL plastic scintillation vials. 6mL of scintillation fluid (Optiphase Safe, Wallac, U.K) was added and the vials capped and shaken for 1-2 hours before being transferred to the liquid scintillation counter (2200CA Tri-Carb LSC, Packard Instruments, U.K.) for the determination of radioactivity .

2.3.9 ^{3}H-dexamethasone standards

The amount of radioligand added to the assay on each experimental day was determined by the inclusion of standards. These consisted of 100μL aliquots of each concentration of ^{3}H-dexamethasone used in the assay and were used for subsequent calculations.

2.3.10. Calculations and analysis of results

Analysis of the data generated using different types of radioligand binding experiments is detailed in the appropriate sections.

2.4. Determination of the saturability of ^{3}H-dexamethasone binding to rat cortical cytosolic preparations

2.4.1. Introduction

Saturation experiments investigate the specific binding of ligand to the receptor at various concentrations of radioligand to obtain estimates for B_{max} (a measure of the receptor concentration in the sample) and K_D (the equilibrium dissociation constant). One of the most important and relevant uses of B_{max} and K_D determinations by saturation binding studies is the estimation of changes in receptor concentration and affinity in disease or following pharmacological or other experimental interventions.

This section aims to demonstrate that the CR assay method used in the present experiments generates reliable saturation binding data relating to the specific binding of ^{3}H-dexamethasone to rat cytosolic tissue preparations.

2.4.2. Methods

Assay procedures were carried out as described in section 2.3. Binding was determined at a range of ^{3}H-dexamethasone concentrations (0.625-20nM). Specific binding was defined in the presence of 5μM hydrocortisone.

2.4.3. Calculations and analysis of results

Radioactivity from saturation experiments (in dpm) was converted to the appropriate units (^{3}H-dexamethasone standards to nM, and TB and NSB incubations to fmoles/assay). Mean values were obtained from the replicate determinations of TB and NSB following which specific ^{3}H-dexamethasone binding was calculated by subtracting NSB values from TB values.

The converted data were subjected to non-linear regression analysis (GraphPad Prism v2.1) to give estimates of B_{max} and K_D following which, B_{max} values were converted to fmoles/mg protein using protein values determined for each of the samples. For the preliminary saturation experiments, the data were also displayed as Scatchard plots with subsequent linear regression analysis, visual inspection of which provided some indication as to whether the ligand-receptor interaction being studied was described adequately by a simple bimolecular reaction. Hill plots of the saturation data enabled the quantitation of deviation of the ligand-receptor interaction from the law of mass action. Differences in binding constants between groups were determined using Students t-test or ANOVA with a significance level set at $p<0.05$.

2.4.4. Results

Figure 2.4.1(A) shows a typical saturation plot of ^{3}H-dexamethasone binding. The non-specific binding of ^{3}H-dexamethasone in rat tissue preparations increased linearly over the ligand concentrations 0.625-20nM. The total and specific binding (defined using 5μM hydrocortisone) of ^{3}H-dexamethasone appeared to saturate at the higher ligand concentrations.

Non-linear regression analysis yielded a B_{max} value of 23.6 fmol/assay, equivalent to a B_{max} of 171 fmol/mg protein. A K_D value of 2.41 nM was obtained from this plot.

Figure 2.4.1(B) shows a Scatchard plot of the data in Figure 2.4.1(A) with subsequent linear regression analysis resulting in a B_{max} of 23.5 fmol/assay equivalent to a B_{max} of 170 fmol/mg protein and a K_D of 2.17 nM.

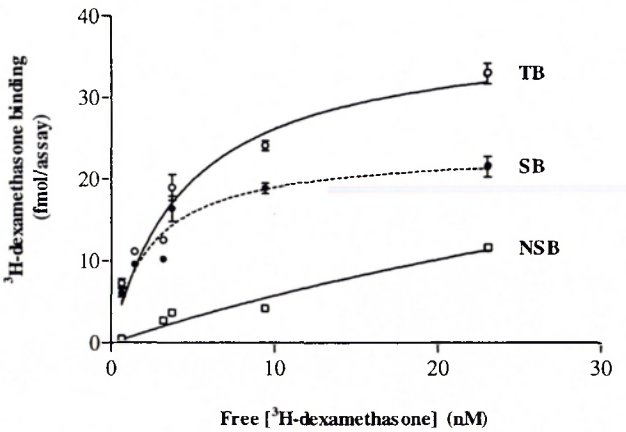
Figure 2.4.1(C) shows a Hill plot of the saturation data from Figures 2.4.1(A) and 2.4.1(B). A Hill coefficient (n_H) of 1.23 ± 0.21 was obtained from this plot.

The results of several saturation assays are shown in Table 2.4.1. Non-linear regression analysis using a one-site binding model resulted in similar values for binding parameters as Scatchard analysis. Statistical analysis of the data, using t-tests showed no significant differences between B_{max} values estimated by non-linear regression and those estimated by Scatchard analysis (whether expressed in fmol/mg tissue or fmol/mg protein). One–way analysis of variance of the K_D data showed no significant differences in K_D values when calculated using non-linear regression, Scatchard or Hill analysis.

The mean B_{max} value calculated from these experiments (using non-linear regression analysis) was 139 ± 12 fmol/mg protein and the mean K_D value calculated was 2.15 ± 0.38 nM.

Figure 2.4.1.

(A)



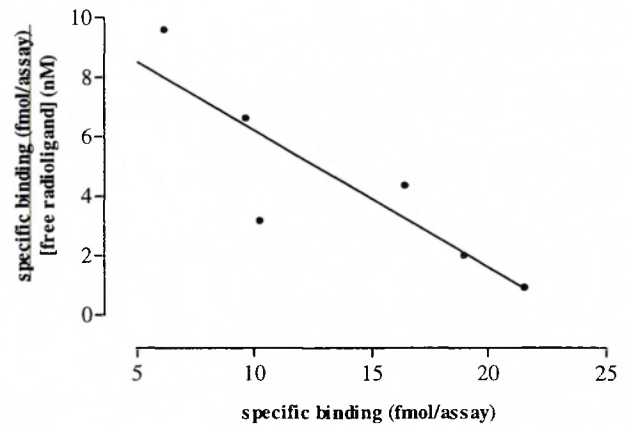
Representative saturation plot of ^{3}H-dexamethasone binding to CR in rat cortical cytosolic preparation.

CR binding assays were carried out as described in section 2.4. Data represent the mean (± standard deviation) for total binding and non-specific binding determinations in duplicate. At a ligand concentration of 3nM, mean (± SD) values for bound in dpm were;

Total binding = 2348 ± 52
Non-specific binding = 533 ± 0
Specific binding = 1815 ± 52
% specific binding = 77.3%

B_{max} = 23.6 fmol/assay
Equivalent to 171 fmol/mg protein
K_D = 2.41 nM

(B)

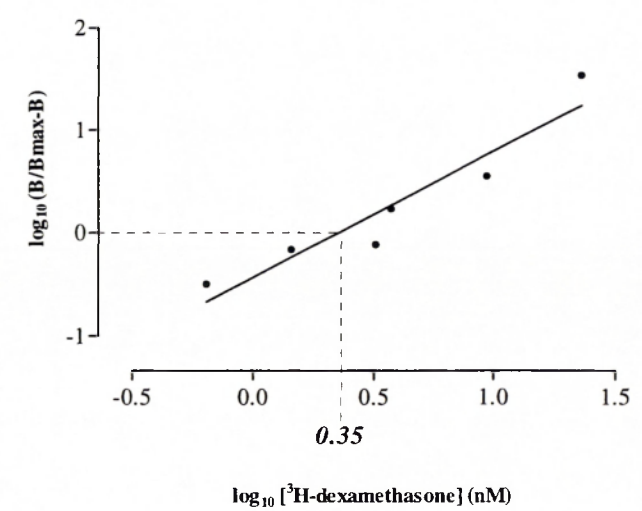


Scatchard plot of ^{3}H-dexamethasone binding to CR in a rat cortical cytosolic preparation (using data shown in Figure 2.3)

Binding parameters (B_{max} and K_D) were calculated using linear regression analysis. Values obtained were;

B_{max} = 23.5 fmol/assay
(converted using protein value=170 fmol/mg protein)
K_D = 2.17 nM

(C)



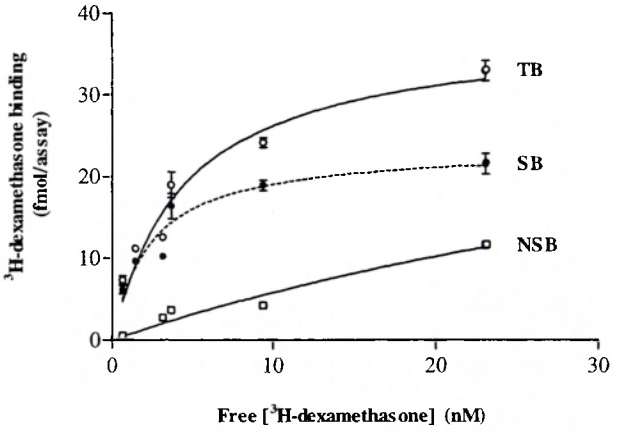
Hill plot of ^{3}H-dexamethasone binding to CR in a rat cortical cytosolic preparation (using data shown in Figure 2.3).

y-axis = *Log10 ($B/(B_{max}-B)$) where;*
B = specific ^{3}H-dexamethasone binding at particular ligand concentration.
B_{max} = B_{max} values using Scatchard analysis.

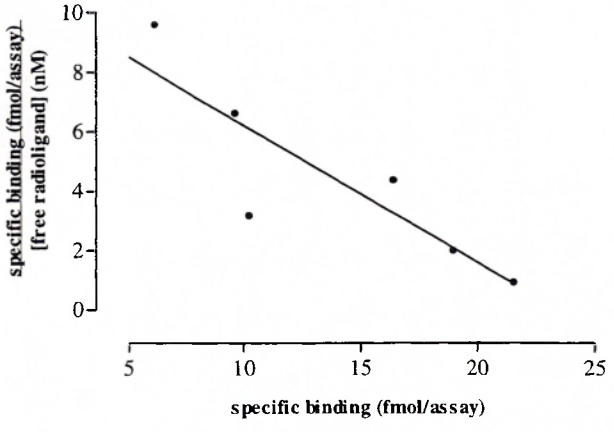
Linear regression analysis was used to calculate the slope of the plot, which corresponds to the Hill coefficient.
Hill coefficient (nH) = 1.23± 0.21
The Hill binding constant (K_D) of ^{3}H-dexamethasone is calculated from the x-axis where log10= ($B/(B_{max})$) = 0
Therefore the **K_D (anti-log = 0.35nM) = 2.24nM**

Figure 2.4.1.

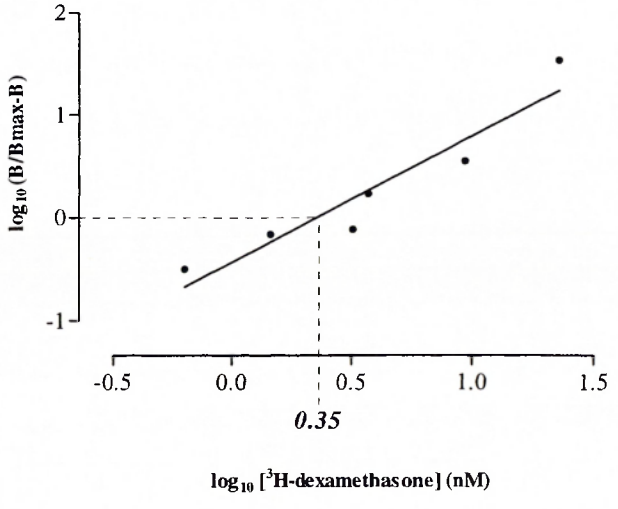
(A)



(B)



(C)



Representative saturation plot of ^{3}H-dexamethasone binding to CR in rat cortical cytosolic preparation.

CR binding assays were carried out as described in section 2.4. Data represent the mean (± standard deviation) for total binding and non-specific binding determinations in duplicate. At a ligand concentration of 3nM, mean (± SD) values for bound in dpm were;

Total binding = 2348 ± 52
Non-specific binding = 533 ± 0
Specific binding = 1815 ± 52
% specific binding = 77.3%

B_{max} = 23.6 fmol/assay
Equivalent to 171 fmol/mg protein
K_D = 2.41 nM

Scatchard plot of ^{3}H-dexamethasone binding to CR in a rat cortical cytosolic preparation (using data shown in Figure 2.3)

Binding parameters (B_{max} and K_D) were calculated using linear regression analysis. Values obtained were;

B_{max} = 23.5 fmol/assay
(converted using protein value=170 fmol/mg protein)
K_D = 2.17 nM

Hill plot of ^{3}H-dexamethasone binding to CR in a rat cortical cytosolic preparation (using data shown in Figure 2.3).

y-axis = *Log10 ($B/(B_{max}$-$B)$) where;*

B = specific ^{3}H-dexamethasone binding at particular ligand concentration.

B_{max} = B_{max} values using Scatchard analysis.

Linear regression analysis was used to calculate the slope of the plot, which corresponds to the Hill coefficient.
Hill coefficient (nH) = 1.23± 0.21
The Hill binding constant (K_D) of ^{3}H-dexamethasone is calculated from the x-axis where log10= ($B/(B_{max})$) = 0
Therefore the **K_D (anti-log = 0.35nM) = 2.24nM**

Table 2.4.1.

Binding parameters obtained from several preliminary saturation experiments conducted in rat cortical tissue preparations (B_{max} is expressed as femtomoles per mg protein and per mg tissue wet weight) using;

1) non-linear regression analysis
2) linear regression analysis subsequent to Scatchard transformation
3) Hill analysis

	NON-LINEAR REGRESSION ANALYSIS			SCATCHARD ANALYSIS			HILL ANALYSIS
	B_{max} (fmol/mg tissue)	B_{max} (fmol/mg protein)	K_D (nM)	B_{max} (fmol/mg tissue)	B_{max} (fmol/mg protein)	K_D (nM)	K_D (nM)
Mean ± sem	3.6 ± 0.42 (n=12)	139 ± 12 (n=12)	2.15 ± 0.38 (n=12)	3.98 ± 0.53 (n=12)	154 ± 18 (n=12)	2.08 ±0.33 (n=12)	2.05 ± 0.29 (n=12)

Data is expressed as mean ± sem for each of the parameters obtained. No statistically significant differences were found between binding parameters obtained using non-linear regression, Scatchard or Hill analysis using t-tests or one-way ANOVA at a significance level of $p<0.05$ (GraphPad Prism v3.0). **ANOVA: CR (*log* K_D)** $F(2, 35) = 0.083$, $p>0.05$.

2.5. Selective displacement of ^{3}H-dexamethasone binding to rat cytosolic preparations using competition binding assays

2.5.1. Introduction

Competition experiments are important in defining the pharmacology of the binding between the ligand and the receptor. Given a single radioligand whose affinity for a particular receptor subtype has been established, radioligand competition assays provide a means for determining the binding affinity of any other unlabelled compound for the same receptor. Applications of the radioligand competition experiment include the validation of assays, screening for identification of ligands for a particular receptor, investigation of the interactions of various compounds with receptors and the determination of receptor density and affinity by use of the same compound as the labelled and unlabelled ligand. Competition experiments measure the binding of a single concentration of radiolabelled ligand in the presence of various concentrations of unlabelled ligands. The generation of an inhibitory constant, IC_{50} (concentration of unlabelled drug that results in 50% displacement of specific binding) or K_i (affinity of the competing compound for the receptor) value is generally the purpose of performing displacement binding assays.

2.5.2. Methods

All assay procedures were carried out as described in section 2.3. Competition studies were carried out at a single concentration of 3nM ^{3}H-dexamethasone. Compounds being tested for competition were added in concentrations ranging from $5x10^{-12}$ to $5x10^{-3}$M. Specific binding was defined using 5μM hydrocortisone. The compounds tested for displacement of ^{3}H-dexamethasone binding to CR were RU28362, dexamethasone, corticosterone, hydrocortisone, aldosterone and ethanol.

2.5.3. Calculations and analysis of results

Mean values were calculated from the replicates and converted to % specific binding. 100% and 0% specific binding was equivalent to the amount of ^{3}H-dexamethasone binding displaced in the absence and presence of 5μM hydrocortisone, respectively. Competing drugs were tested over a range of concentrations for their capacity to compete for this specific binding. The data was plotted on a semilogarithmic plot resulting in a displacement curve and an IC_{50} value was determined by non-linear regression analysis (GraphPad Prism v3.0). This was subsequently used to calculate the inhibition constant (K_i) for each unlabelled compound using the equation of Cheng and Prusoff (1973). K_i values are expressed in Table 2.5.1 as geometric means of several determinations as, due to the log-normal distribution of drug affinities, these appear to be best described in this manner rather than by using arithmetic means (Hancock *et al*, 1988).

2.5.4. Results

Table 2.5.1 summarises the competition experiments for each compound tested while Figure 2.5.1 displays representative competition experiments for each compound. All of the steroids tested competed for specific ^{3}H-dexamethasone binding to rat cytosolic fractions in a concentration-dependent manner. The affinity of competitors for ^{3}H-dexamethasone binding to CR ranged from 0.018 – 6.03nM with the following rank order of potency; RU28362 > dexamethasone > corticosterone > hydrocortisone > aldosterone. There was no displacement of specific binding by EtOH.

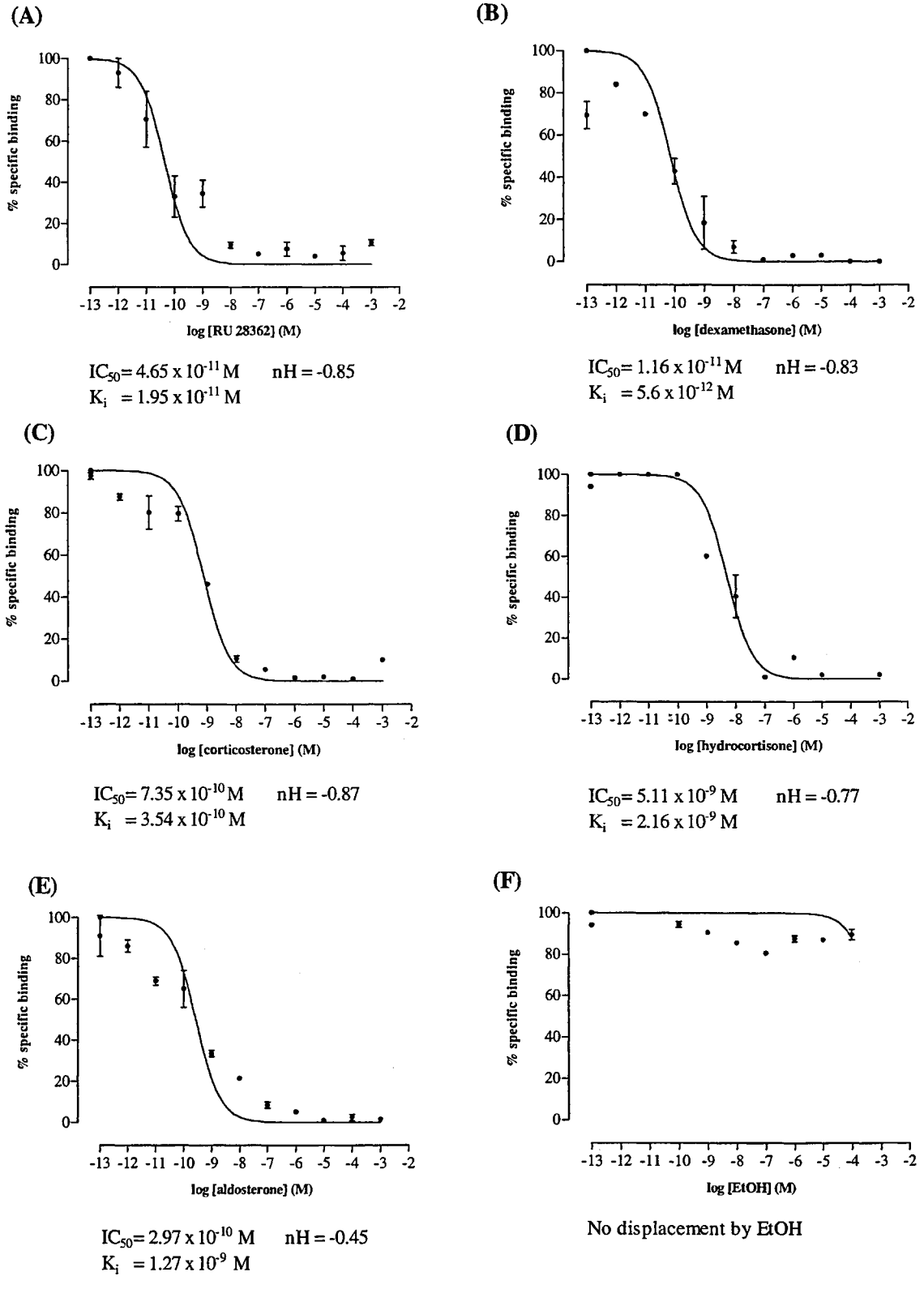
Table 2.5.1.

Displacement of specific ^{3}H-dexamethasone binding to CR in rat cytosolic preparations by RU 28362, dexamethasone, corticosterone, hydrocortisone, aldosterone and ethanol. Summary of values obtained from experiments testing the displacement of specific ^{3}H-dexamethasone binding to CR in rat cytosolic preparations by various compounds. Data from four independent experiments conducted on separate occasions were fitted to one-site competition curves resulting in estimations of IC_{50} (concentration of unlabelled compound which causes 50% displacement of maximal binding) from which K_I (affinity of the competing compound for the receptor) was calculated using the Cheng-Prusoff equation for each compound. The K_D value of ^{3}H-dexamethasone binding obtained in parallel saturation experiments used in these calculations was 2.5nM. K_I is expressed as a geometric mean with numbers in parentheses indicating the range for each value.

COMPOUND TESTED FOR DISPLACEMENT	K_I (nM)
RU 28362	**0.018** (0.0068 – 0.049)
Dexamethasone	**0.87** (0.48 – 1.58)
Corticosterone	**3.16** (1.15 –8.69)
Hydrocortisone	**4.83** (2.06 – 11.32)
Aldosterone	**6.03** (3.23 – 11.2)
EtOH	-

Figure 2.5.1.

Displacement of specific ^{3}H-dexamethasone binding from CR in rat cytosolic preparations by RU28362 (A), dexamethasone (B), corticosterone (C), hydrocortisone (D), aldosterone (E) and EtOH (F). Rat cytosolic fractions were prepared as described in section 2.5.2. Data shown is from a single experiment.



2.6. Determination of the relationship between specific ^{3}H-dexamethasone binding and concentration of rat cortical cytosol tissue

2.6.1. Introduction

The establishment of a linear relationship between specific binding and tissue concentration in binding experiments is important to demonstrate the absence of artefacts in the assay (such as receptor or ligand degradation, the presence of unrecognised endogenous ligands) thus ensuring the accurate estimation of binding parameters. The tissue dilution used in the assay must also yield measurable specific binding values which give a good 'experimental gate' to work with. In order to prevent the above problems, the tissue concentration used in the assay should bind no more than 10% of the added radioligand.

2.6.2. Methods

Rat cortical cytosol fractions were prepared as described in section 2.3.5 except that cortical tissue was homogenised in 1:5 w/v of incubation buffer and following centrifugation, the tissue supernatant was diluted to the required concentration. All other assay procedures were carried out as described in section 2.3.

2.6.3. Calculations and analysis

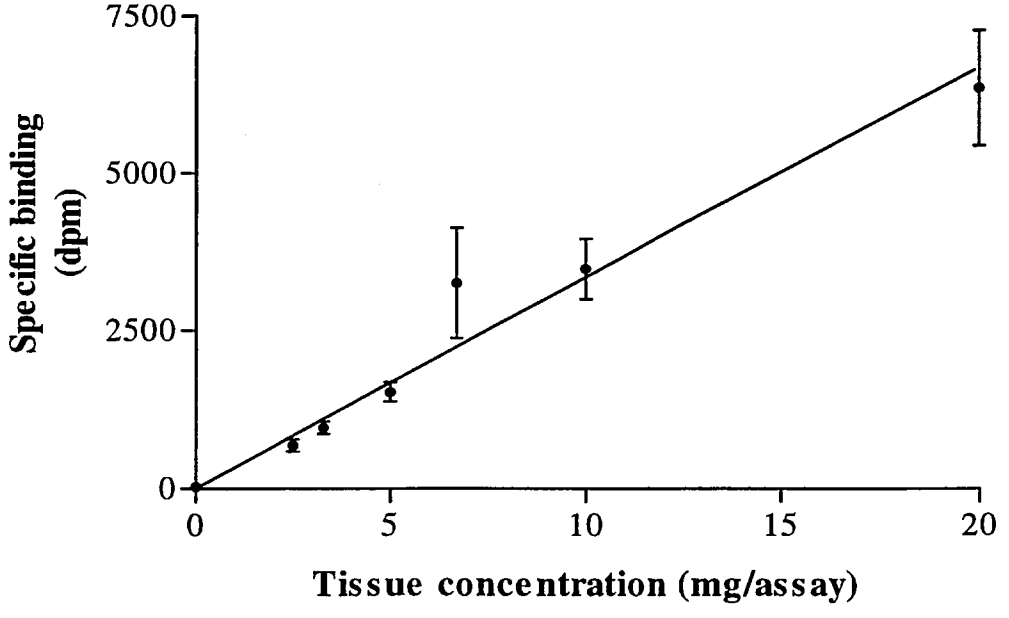
The specific binding of ^{3}H-dexamethasone to CR in the cytosol preparation was calculated by subtracting NSB from TB and plotted against tissue concentration. Linear regression analysis was performed (using GraphPad Prism v3.0) to determine the effects of tissue concentration on the specific binding of ^{3}H-dexamethasone.

2.6.4. Results

Specific binding of ^{3}H-dexamethasone increased linearly with increasing tissue concentration (Figure 2.6.1). The values of specific binding observed at tissue concentrations of 5 and 3.3 mg wet weight original tissue were 1523 and 962 dpm (corresponding to 9.3 and 8.8 fmol/mg protein) respectively, thus giving measurable values for the specific binding of ^{3}H-dexamethasone at these concentrations of tissue. The mean values for added ^{3}H-dexamethasone in these experiments was 175990 ± 6101 dpm at 3nM of ^{3}H-dexamethasone and the amount of radioactivity bound expressed as a percentage of added radioactivity was approximately 1%.

FIGURE 2.6.1.

The effect of tissue concentration on specific binding of ^{3}H-dexamethasone binding to CR in rat cortical cytosolic preparations.



Rat cytosolic fractions prepared using a 1:5 w/v homogenisation volume were prepared as described in section 2.3. The resulting supernatant was diluted to give cytosolic fractions at tissue concentrations of 2.5 - 20mg wet weight of tissue/incubation. Assay procedures were carried out as described in section 2.4. Data shown represents the mean (± s.e.m) for five experiments.

2.7. Effect of incubation temperature on the specific binding of ^{3}H-dexamethasone to rat cortical cytosol

2.7.1. Introduction

The incubation temperature of binding assays can strongly affect the rates of association and dissociation (see section 2.8) and may affect equilibrium dissociation constants. The following experiments were designed to optimise the incubation temperature for the CR binding assays.

2.7.2. Methods

All assay procedures were carried out as described in section 2.3 except that assay tubes were incubated at temperatures of 4°C, 21°C and 32°C for 24 hours.

2.7.3. Calculations and analysis

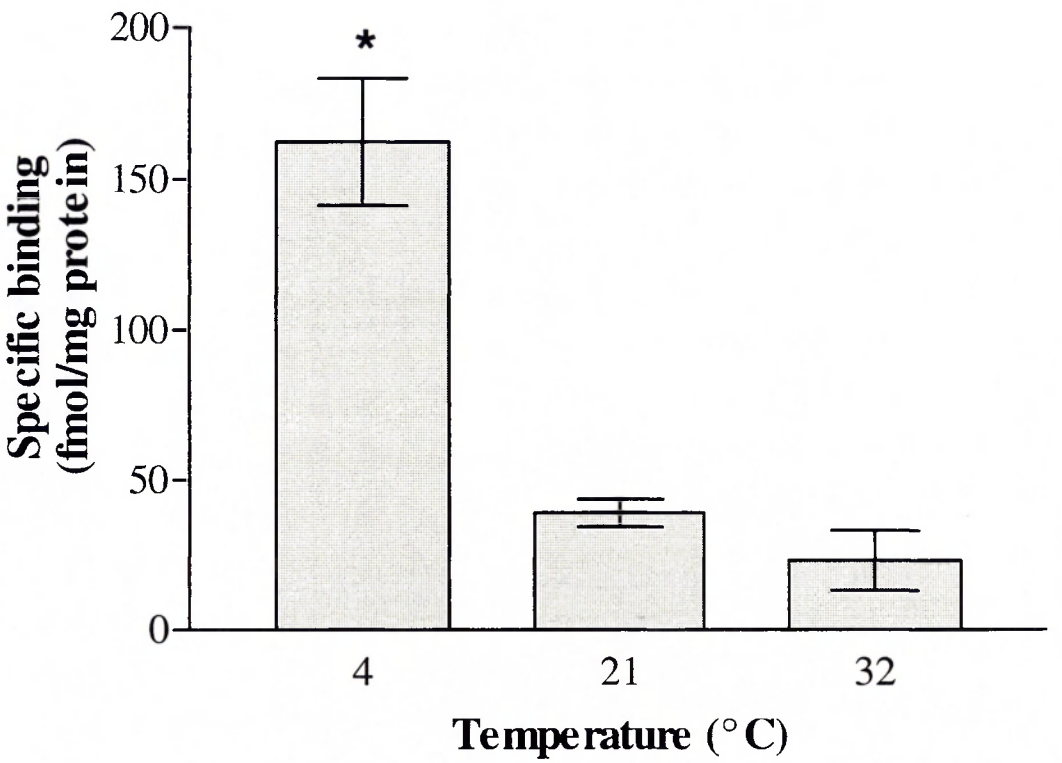
Specific ^{3}H-dexamethasone binding was determined and plotted against temperature. One-way ANOVA (using GraphPad Prism v3.0) was used to test the significance of any differences in specific ^{3}H-dexamethasone binding to CR in rat cytosol.

2.7.4. Results

At temperatures of 32°C and 21°C, there was very little specific binding of ^{3}H-dexamethasone to tissue fractions (23.1 and 39 fmol/mg protein respectively – see Figure 2.7.1). Specific ^{3}H-dexamethasone binding to CR was highest at 4°C (162.3 fmol/mg protein). ANOVA and subsequent post-hoc analysis using the Newman-Keuls test showed a significant difference ($p<0.001$) between specific ^{3}H-dexamethasone binding at 4°C as compared to binding at 21°C and 32°C.

FIGURE 2.7.1

The effect of incubation temperature on specific binding of ^{3}H-dexamethasone binding to CR in rat cortical cytosolic preparations.



Rat cortical cytosolic fractions (prepared as described in section 2.3.5) were incubated with 3nM ^{3}H-dexamethasone for 24 hours at various temperatures (32°C, 21°C and 4°C). The assay was carried out as described in section 2.3. Data shown represents the mean (± s.e.m) for four experiments. Statistically significant differences between groups were determined using one-way ANOVA followed by subsequent Newman-Keuls post-hoc analysis and are denoted by *$p<0.01$. **ANOVA** – $F(2, 11) = 31.34$, $p<0.0001$.

2.8. Kinetics experiments: association and dissociation

2.8.1. Introduction

Main objectives for performing kinetics experiments are to;

i) establish when steady state is reached so that saturation and inhibition experiments can be accurately performed

ii) determine the association rate constant (k_{+1}) and dissociation rate (k_{-1}) constant of the reaction so that the K_D can be subsequently calculated.

Kinetics experiments produce a useful independent estimate of the K_D and therefore aid in the design of the assay with regards to methods of separation. K_D values obtained using these calculations also provide a check on the internal consistency of the receptor–ligand system under investigation.

2.8.2. Methods

Assay procedures were carried out as described in section 2.3. In association experiments, tissue supernatant was incubated with 3nM ^{3}H-dexamethasone over varying periods of time up to 48 hours.

In dissociation experiments, cytosolic fractions were incubated with 3nM ^{3}H-dexamethasone at 4°C for 24 hours to achieve binding equilibrium (this time being determined by the association experiments). At 24 hours of incubation, sets of test tubes were filtered to determine TB (in triplicate) and NSB (in triplicate) at equilibrium. 50µL of hydrocortisone (25µM) was added to the remaining incubations to occupy all the free receptors thereby blocking the associative reaction so that only the dissociation reaction was measured. Individual sets of test tubes were filtered at various time intervals following the addition of hydrocortisone (1–25 hours).

2.8.3. Calculations and analysis

The specific binding of ^{3}H-dexamethasone was plotted against time. Non-linear regression analysis (GraphPad Prism v3.0) was used to fit the data to a one-phase exponential association equation. A pseudo 1st order association plot of the data was constructed and followed by linear regression analysis in order to calculate the parameter k_{obs} (k observed).

The rate constant for the dissociation reaction rate was calculated by non-linear regression analysis using the one-phase exponential decay equation (GraphPad Prism v3.0).

The association rate constant (k_{+1}) was calculated by substituting these values into the following equation;

$$k_{+1} = \frac{(k_{obs} - k_{-1})}{F} \qquad \text{where F = free ligand concentration}$$

Substitution of the k_{-1} and k_{+1} values obtained into the following equation resulted in a value for the dissociation constant (K_D).

$$K_D = k_{-1} / k_{+1}$$

2.8.4. Results

Figure 2.8.1 (A) shows the mean (± sem) association data from five experiments. The association of ^{3}H-dexamethasone to cortical preparations was slow ($t_{1/2}$=6.4 hours) and achieved equilibrium after 24 hours of incubation. Binding equilibrium was maintained up to 48 hours of incubation. The parameter k_{obs} (k observed) was calculated by a pseudo 1st order association plot of the data (see Figure 2.8.1 (B)). Analysis of this data yielded a value of 0.11 min^{-1} for k_{obs}.

Figure 2.8.1 (C) shows the mean (± sem) dissociation data from three experiments. The dissociation of ^{3}H-dexamethasone from its binding sites was also very slow with a $t_{½}$= 17.55 hours. The rate constant (calculated by non-linear regression analysis) for the dissociation reaction rate (k_{-1}) was 0.0395 min^{-1}.

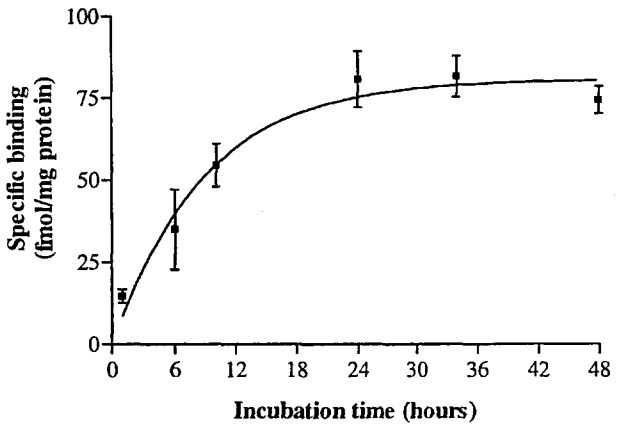
A value of 0.0169 min^{-1} nM^{-1} was calculated for the association rate constant (k_{+1}). Substituting these values into the following equation;

$$K_D = k_{-1} / k_{+1}$$

resulted in a calculated K_D value of 2.34nM.

Figure 2.8.1.

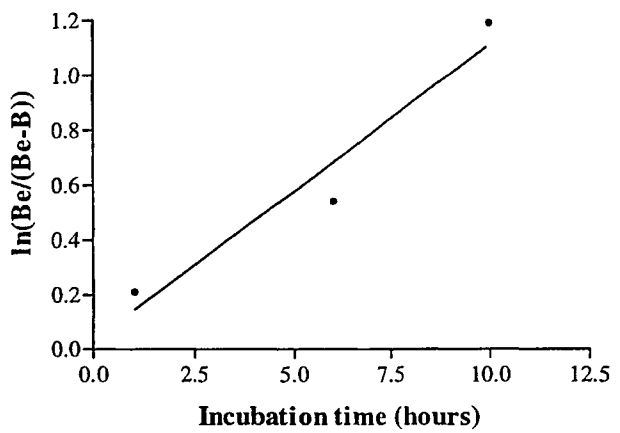
(A)



Time course of association of specific binding of ^{3}H-dexamethasone binding to CR in rat cortical cytosolic preparations.

Assay procedures were carried out as described in section 2.8.2. Data shown represents the mean (± s.e.m) for five experiments. Equilibrium was achieved at 24 hours of incubation at 4°C. Specific binding after 24 hours of incubation at 4°C was 8.07 ± 0.86 fmol/mg protein.
Non-linear regression analysis was used to fit the data to a one-phase exponential association equation (see section 2.8.3).

(B)



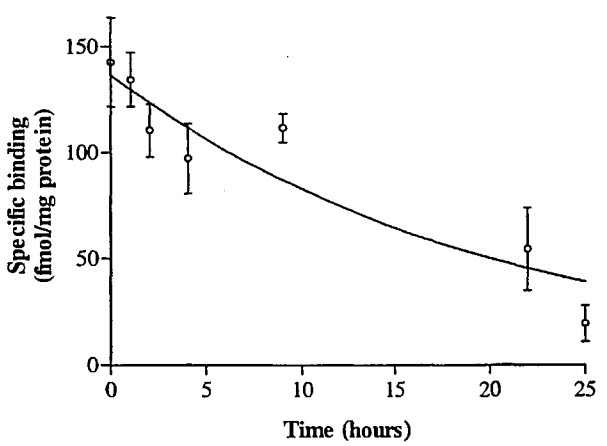
Pseudo first order association plot

Y-axis : *ln (Be / (Be-B))*

where; *Be* = binding at equilbrium
B = binding at a particular time

This plot was constructed (see section 2.8.3) in order to calculate the rate constant (k_{obs}). The value for k_{obs} was obtained from the slope of the plot shown here and was 0.112 ± 0.01min-1.

(C)



Time course of dissociation of specific binding of ^{3}H-dexamethasone binding to CR in rat cortical cytosolic preparations.

Assay procedures were carried out as described in section 2.8.2. Data shown represents the mean (± s.e.m) for three experiments. Equilibrium was achieved at 24 hours of incubation at 4°C following which the association reaction was blocked by the addition of 5µM hydrocortisone. Specific binding after 24 hours of incubation at 4°C was 14.26 ± 2.12 fmol/mg protein. Specific binding at 25 hours after the termination of the association reaction was 1.96 ± 0.85 fmol/mg protein. Non-linear regression analysis was used to fit the data to a one-phase exponential decay equation.

2.9. The determination of specific ^{3}H-dexamethasone binding to rat cytosolic preparations from fresh and frozen tissue

2.9.1. Introduction

This investigation examined the effects of freezing tissue samples at -70°C on the binding parameters of ^{3}H-dexamethasone. This study was important in relation to future large studies that were being planned, as many of the samples would require freezing for some period of time prior to being assayed.

2.9.2. Methods

This study was carried out in both cortical and hippocampal tissues dissected from rat brain (as described in section 2.3.2) and frozen for periods of 1, 3, 6 and 12 months at -70°C. Fresh tissue was also dissected on each experimental day and assayed in parallel, to act as the control sample. Assay procedures were carried out as described in section 2.3. except that the separation of free and bound ^{3}H-dexamethasone was carried out using a 12-hole Millipore manifold system.

2.9.3. Calculations and analysis

Saturation data obtained from these experiments was analysed as described in section 2.4.3. One-way analysis of variance (GraphPad Prism v2.1) was applied to the data to determine any differences in the binding parameters of fresh, 1, 3, 6 and 12 month frozen samples.

2.9.4. Results

ANOVA revealed no significant differences in the binding parameters of specific ^{3}H-dexamethasone to CR in fresh cortex over 12 months ($p>0.05$) therefore these were pooled to give overall values of 177 ± 11 fmol/mg protein (n=24) for B_{max} and 2.36 ± 0.26 nM (n=24) for K_D. These pooled fresh values were used in subsequent statistical analysis. B_{max} values of specific ^{3}H-dexamethasone binding to CR were not significantly different from fresh values in 1, 3 and 12 month frozen cortex however a significant difference was observed between fresh and 6 month frozen values (Table 2.9.1). There was no difference in K_D values in fresh or frozen cortex.

Tables 2.9.2. (A) and (B) show the B_{max} and K_D of specific ^{3}H-dexamethasone binding to fresh hippocampal tissue preparations, which were consistent in assays performed over a 12 month period. These values were pooled giving overall values of 130 ± 8 fmol/mg protein for B_{max} and 2.21 ± 0.41 nM for K_D which were used in subsequent statistical analysis. No significant differences were observed following ANOVA and post-hoc tests in B_{max} and K_D values for 1, 6 and 12 month frozen hippocampal tissue as compared with pooled fresh values.

Table 2.9.1. B_{max} (A) and K_D (B) values of total CR obtained in cytosolic preparations from fresh and frozen rat cortex.

(A) B_{max}

FROZEN CORTEX				
FRESH CORTEX	**1 MONTH**	**3 MONTH**	**6 MONTH**	**12 MONTH**
179 ± 14 (n=6)	133 ± 15 (n=6)	---	---	---
173 ± 16 (n=10)	---	152 ± 8 (n=10)	---	---
171 ± 15 (n=5)	---	---	96 ± 10 * (n=5)	---
193 ± 44 (n=5)	---	---	---	154 ± 37 (n=5)

(B) K_D

FROZEN CORTEX				
FRESH CORTEX	**1 MONTH**	**3 MONTH**	**6 MONTH**	**12 MONTH**
2.91 ± 0.71 (n=6)	2.62 ± 0.59 (n=6)	---	---	---
2.13 ± 0.44 (n=10)	---	1.65 ± 0.24 (n=10)	---	---
1.87 ± 0.4 (n=5)	---	---	1.44 ± 0.22 (n=5)	---
3.25 ± 0.32 (n=5)	---	---	---	4.47 ± 1.88 (n=5)

CR binding assays were conducted in frozen cortical tissue (with fresh samples assayed in parallel) as described in section 2.3. Total binding determinations were made in duplicate. Non-specific binding was defined in single tubes in the presence of 5μM hydrocortisone. Data represent the mean ± sem values from the number (n) of samples indicated. One-way ANOVA revealed no significant differences between fresh samples at 1, 3, 6 and 12 months *(**ANOVA-fresh CR (B_{max})** $F(3, 25) = 0.17$, $p>0.05$; **CR (log K_D)** $F(3, 25) = 1.66$, $p>0.05$)* therefore these were pooled (giving a value of 177 ± 11 fmol/mg protein (n=24) for B_{max} and 2.36 ± 0.26 nM (n=24) for K_D) which were used in subsequent analysis. Data were further analysed using ANOVA followed by Newman Keuls tests (GraphPad Prism v3.0). ***ANOVA: CR (B_{max})*** *$F(4, 49) = 3.17$, $p<0.05$.* ***CR (log K_D)*** *$F(4, 49) = 1.82$, $p>0.05$. Significant differences between the pooled fresh values and frozen values are denoted by* $p<0.05$*

Table 2.9.2. B_{max} (A) and K_D (B) values of total CR obtained in cytosolic preparations from fresh and frozen rat hippocampus.

(A) B_{max}

FROZEN HIPPOCAMPUS			
FRESH HIPPOCAMPUS	**1 MONTH**	**6 MONTH**	**12 MONTH**
141 ± 8 (n=5)	147 ± 13 (n=5)	---	---
143 ± 14 (n=4)	---	108 ± 6 (n=4)	---
111 ± 17 (n=5)	---	---	110 ± 21 (n=5)

(B) K_D

FROZEN HIPPOCAMPUS			
FRESH HIPPOCAMPUS	**1 MONTH**	**6 MONTH**	**12 MONTH**
1.57 ± 0.17 (n=5)	1.5 ± 0.21 (n=5)	---	---
2.22 ± 0.6 (n=4)	---	1.3 ± 0.06 (n=4)	---
2.83 ± 1.05 (n=5)	---	---	3.7 ± 0.52 (n=5)

CR binding assays were conducted in frozen hippocampal tissue (with fresh samples assayed in parallel) as described in section 2.3. Total binding determinations were made in duplicate. Non-specific binding was defined in single tubes in the presence of 5μM hydrocortisone. The data represent the mean ± sem values from the number (n) of samples indicated. One-way ANOVA revealed no significant differences between fresh samples at 1, 6 and 12 months *(**ANOVA-fresh CR (B_{max})** $F(2, 13) = 1.77$, $p>0.05$; **CR (log K_D)** $F(2, 13) = 0.27$, $p>0.05$)* therefore these were pooled (giving a value of 130 ± 8 fmol/mg protein (n=14) for B_{max} and 2.21 ± 0.41 nM (n=14) for K_D) which were used in subsequent analysis. Data were further analysed using ANOVA followed by Newman Keuls tests (using GraphPad Prism v3.0). ***ANOVA: CR (B_{max})*** $F(3, 27) = 1.63$, $p>0.05$. ***CR (log K_D)*** $F(3, 27) = 3.25$, $p<0.05$. No significant differences in B_{max} were found between the pooled fresh values and frozen values. ANOVA showed significant effects of freezing on K_D values however, subsequent post-hoc analysis between pooled fresh and frozen values did not display any significant differences.

2.10. Determination of total CR (type I + II receptors) population and GR (type II receptors) using RU28362

2.10.1. Introduction

This study aims to investigate the specific binding of ^{3}H-dexamethasone to type I (MR) and type II (GR) populations in rat cortical preparations. The wide distribution of type II GR in the brain should make their detection in the cortex relatively simple and the different types of receptor can be distinguished from one another using synthetic, selective glucocorticoid agonists such as RU28362 (11β, 17β-dihydroxy-6-methyl-17α(1-propynyl)–androsta-1, 4, 6-trione-3-one) or RU26988 (11β, 17β-dihydroxy-17α-(1-propynyl)–androsta-1, 4, 6-trione-3-one).

2.10.2. Methods

Assay procedures were carried out as described in section 2.3. In addition to the total binding and non-specific binding tubes, another set of test tubes was added to the assay for determinations of specific ^{3}H-dexamethasone binding in the presence of the selective GR agonist, RU 28362 (0.5μM or 100 times in excess of ^{3}H-dexamethasone concentration), which were made singly.

2.10.3. Calculations and analysis

Specific ^{3}H-dexamethasone binding to total CR was calculated. Specific ^{3}H-dexamethasone binding to type II GR was calculated by subtracting the binding in the presence of RU28362 from the total binding.

Saturation data obtained from these experiments was analysed as described in section 2.4.3. One-way analysis of variance (using GraphPad Prism v3.0) at a significance level of <0.05 was applied to the data to determine any differences in the specific binding of ^{3}H-dexamethasone when being displaced by RU28362;

1) at a fixed concentration of 0.5μM or

2) at a concentration 100 times in excess of ^{3}H-dexamethasone in the assay.

2.10.4. Results

Table 2.10.1 shows the binding parameters obtained for total CR population, type II GR measurement using RU 28362 at a fixed concentration and at concentrations 100 times in excess of ^{3}H-dexamethasone in the assay. One–way analysis of variance showed no difference between the B_{max} and K_D of total CR, type II GR defined using a fixed concentration of RU28362 and type II GR using RU28362 100x in excess of ^{3}H-dexamethasone.

Table 2.10.1

Binding parameters of total CR (type I MR + type II GR) population and type II (GR) receptors in rat cortex using fixed and variable concentrations of RU28362.

PARAMETER	Type I + II CR	Type II GR (fixed - 0.5μM RU28362)	Type II GR (100x excess RU28362)
B_{max} (fmol/mg protein)	**237 ± 32** (n = 4)	**219 ± 28** (n = 4)	**233 ± 34** (n=4)
K_D (nM)	**1.43 ± 0.6** (n = 4)	**1.17 ± 0.43** (n = 4)	**1.32 ± 0.47** (n = 4)

Assay procedures were carried out as described in section 2.3. Type II GR were defined using RU 28362 at a fixed concentration of 0.5μM and at concentrations that were 100 times in excess of [^{3}H-dexamethasone] in the assay. Statistically significant differences between groups were determined using one-way ANOVA followed by subsequent Newman-Keuls post hoc analysis. ANOVA revealed no significant differences between B_{max} ($F(2, 11) = 0.094$, $p>0.05$) or K_D ($F(2, 11) = 0.039$, $p>0.05$) values obtained for total CR population and type II GR population in rat cortex.

2.11. Protein determination using the Lowry method

2.11.1. Introduction

Protein content in tissue samples was estimated using the colorimetric method of Lowry *et al* (1951). This method utilises hydrolytic reactions to break the protein down into its constitutive amino acids. A coloured complex results from interactions between an alkaline copper–phenol reagent and tyrosine and tryptophan residues in the protein. The protein content of the sample is estimated via a spectrophotometric reading of absorbance.

2.11.2. Methods

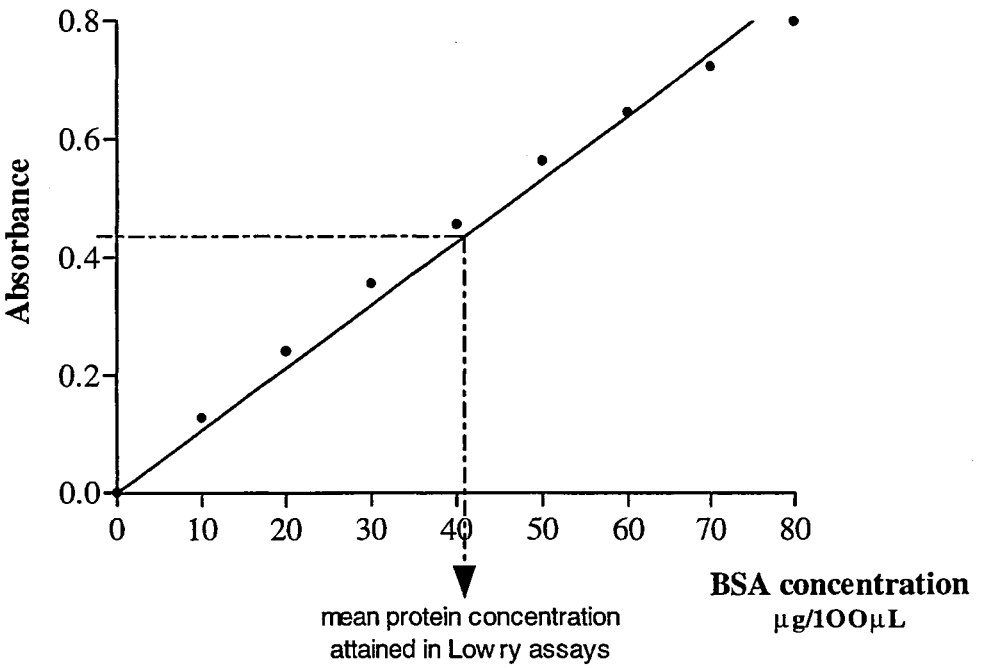
The Lowry protein assay was adapted from the standard protocol (Lowry *et al,* 1951). The presence of Tris and glycerol as components of the incubation buffer used in the CR assay, posed a problem. These two compounds are found to interfere with the measurement of protein by the Lowry method by contributing blank colour and/or decreasing chromophore development with protein (Rej *et al,* 1974). This distortion was overcome by diluting the tissue samples with distilled water (1:4) therefore effectively diluting out the interfering components. Incorporation of the same amounts of these compounds in the standards also overcame the problems we encountered. The standard curve was therefore constructed in incubation buffer that had been diluted with distilled water by the same factor as the samples for the protein assay.

2.11.3. Results

Standard curves obtained in the Lowry protein assay were linear over the range of 10-80μg BSA (see Figure 2.11.1).

Figure 2.11.1.

Protein determination using the Lowry method



Standard curve and mean protein concentration obtained using the Lowry assay. Standard curves were linear over the range of 10-80µg BSA. Average values in our studies were 41µg/100µL volume (160 µg/assay when multiplied by 4, the dilution factor for the tissue samples).

2.11.4. Protein determination using Lowry method

Protein values obtained using this method were very consistent. Dilution of the samples and construction of the standard curve in diluted incubation buffer overcame the problem of interfering compounds such as Tris and glycerol.

2.12. Discussion

2.12.1. Determination of the saturability of ^{3}H-dexamethasone binding to CR in rat cortical cytosolic preparations

Binding parameters in these experiments were determined using linear and non-linear regression analysis. B_{max} and K_D values obtained using both of these methods were in close agreement suggesting that the CR assay satisfies the required criteria for receptor binding experiments. The determination of binding parameters using linear regression analysis however is questionable due to the distortion of experimental error and alteration of values that occurs when performing linear transformations on saturation data (Motulsky, 1996). Displaying saturation data as Scatchard and Hill plots was useful however in determining whether or not the ligand-receptor interaction was described adequately by a simple bimolecular reaction and enabling the quantitation of deviation of the ligand-receptor interaction from the law of mass action. Non-linear regression was chosen as the standard method of analysis for saturation data as this is the statistically correct method. This type of analysis also minimises the contribution of outliers by introducing appropriate weighting whereas linear regression gives equal weighting to all the points.

Analysis of saturation binding data using non-linear regression is based on the following assumptions;

1) binding follows the law of mass action
2) there is only one population of receptors
3) there is no co-operativity between binding sites (i.e. constant)

Results of the CR saturation binding experiments showed that;

1) Specific ^{3}H-dexamethasone binding to rat cortical cytosol is saturable and follows the law of mass action (as indicated by a Hill coefficient close to unity).
2) ^{3}H-dexamethasone is binding to a single binding site as shown by a Hill coefficient close to 1.
3) there is no cooperativity between sites as shown by linearity of the Scatchard plot and a Hill coefficient close to unity. The reaction between the receptor and its ligand is a simple, bimolecular reaction.

The interaction between ^{3}H-dexamethasone and its binding site therefore appeared to follow the assumptions made when performing saturation analysis using non-linear regression analysis.

Non-linear regression analysis of cortical CR binding data gave B_{max} and K_D values that ranged from 60-198 fmol/mg protein and 0.79-6.33 nM, respectively. The mean values for B_{max} and K_D (139 fmol/mg protein and 2.15 nM, respectively) calculated from these saturation experiments provided a guideline for binding parameters which were useful in future studies. These values also appear to be in agreement with those obtained for type II GR in various brain regions in previous studies (Reul *et al*, 1985; Allen *et al*, 1988; Spencer *et al*, 1990; Reul *et al*, 1993; 1994 and Budziszweska *et al*, 1994a). Mean values in these investigations were in the range of 50-200 fmol mg protein for Bmax (higher B_{max} values were obtained in studies utilising adrenalectomised animals) and 0.4-2.0 nM for K_D.

The agreement of our data with that of other groups indicates that the binding of ^{3}H-dexamethasone to CR in rat brain as assessed by this assay system is a viable method of

measuring CR. It appears that the components of the assay buffer which include chelating agents and sulfhydryl moeties, are in proportion and allow stabilisation of the CR which, when unoccupied are generally very unstable in vitro. The physical conditions of the assay such as temperature and time of incubation have been chosen to allow optimal binding of ^{3}H-dexamethasone to CR. The use of the rapid vacuum filtration technique as a method of separation of bound and free ligand is also thereby supported as previous investigations have used column and dextran-charcoal separation methods.

2.12.2. Selective displacement of ^{3}H-dexamethasone binding to rat cortical cytosolic preparations.

EtOH did not compete for specific ^{3}H-dexamethasone binding to CR in rat cytosol preparations. Competing compounds used in the CR binding assay were dissolved in absolute ethanol and subsequently diluted in 10% EtOH (2% final assay concentration) and it was important to be certain that there was no interaction of the CR with this component of the assay. Results of the competition experiments showed relative binding affinities (RBA) for CR as follows; RU28362 > dexamethasone > corticosterone > hydrocortisone > aldosterone.

Hydrocortisone competition experiments gave a K_i value of 4.8nM. This value aided in determining the concentration of hydrocortisone to be used as the displacing compound for non-specific binding determinations in the CR assay. A concentration of 5μM (final assay) was chosen for non-specific binding as this concentration is over 1000 times the K_I of hydrocortisone for CR and will displace up to 99% of the specifically bound radioligand.

In experiments using RU 28362 to define ^{3}H-dexamethasone binding to type II CR (see section 2.10), the concentration of RU 28362 was determined by the competition experiments for this compound. A K_i value of 0.018 nM was obtained from the competition curve for RU 28362. As a result of these experiments, a concentration of 0.5μM was chosen for type II CR definition in the assay. The potency of RU28362 for ^{3}H-dexamethasone displacement from CR binding sites suggests that this compound may not be as selective for type II CR binding as was previously thought. Previous studies have shown that RU28362 competes for ^{3}H-dexamethasone binding to type I as well as type II CR, but not for ^{3}H-corticosterone binding to type I CR (Jacobson *et al*, 1993). However, not all other investigators have observed similar results with ^{3}H-dexamethasone (Spencer *et al*, 1991) therefore this issue remains unclear.

Competition profiles in these experiments were similar to those obtained previously by other laboratories with RU28362 as the most potent competitor for ^{3}H-dexamethasone binding (Vedder *et al*, 1993). This was followed by dexamethasone and aldosterone thus revealing a competition profile characteristic of type II CR. Competition analysis of ^{3}H-dexamethasone binding sites showed that the majority of labelled receptors were type II CR.

2.12.3. Determination of the linear relationship between specific ^{3}H-dexamethasone binding and rat cytosolic tissue concentration

These experiments demonstrated that the use of this assay to measure the binding parameters of CR in rat cytosolic preparations yields consistent results, which are free from the artefacts mentioned in section 2.6.1. A tissue concentration of 5mg wet weight original tissue was chosen from the linear portion of the tissue linearity graph (Figure 2.2) and was employed routinely for subsequent experiments except where mentioned.

These experiments also ensured that measurable specific ^{3}H-dexamethasone binding to CR was obtained at the concentration of tissue chosen in the assay and that no more than 10% of added radioligand was bound by the concentration of tissue chosen for subsequent experiments.

2.12.4. Effect of incubation temperature on the specific binding of ^{3}H-dexamethasone to rat cortical cytosolic preparations

From the results obtained in these experiments, it was decided to proceed with the optimum incubation temperature of 4°C for all subsequent CR binding assays. This result was expected as CR are generally very unstable and deactivate at high temperatures due to the disruption of the complex structure of receptor protein.

2.12.5. Kinetics experiments (association and dissociation)

The association experiments showed that the incubation time chosen for the CR binding assay (approximately 24 hours) is sufficient for the receptor-ligand interaction to reach equilibrium in saturation and competition experiments. The linearity of the pseudo-first order association plot demonstrates that the interaction of the receptor and radioligand can be described by a simple bimolecular reaction (McGonigle & Molinoff, 1989).

The dissociation of the receptor-ligand complex appears to be a relatively slow process therefore the filtration method of separation used in the CR assay is rapid enough to prevent excessive loss of specific ^{3}H-dexamethasone binding.

The K_D value of ^{3}H-dexamethasone binding to CR obtained by performing these kinetics experiments was in close agreement to K_D values obtained in saturation

experiments (section 2.4) thereby providing further support for the reliability of estimates produced using the saturation experiments.

2.12.6. The determination of specific ^{3}H-dexamethasone binding to rat cytosolic preparation from fresh and frozen tissue

Assays carried out in these experiments were initially filtered using a 12-hole manifold system therefore in order to keep the method of filtration consistent, the Millipore apparatus was used for all of the samples. However, preliminary experiments comparing the binding parameters obtained using the Millipore system and Brandel Cell Harvester showed no differences in B_{max} and K_D values in tissues that have been filtered through the Millipore or Brandel systems.

The results of these experiments showed there were few differences in B_{max} or K_D values obtained in fresh and frozen samples taken from different brain regions. B_{max} values were reduced in 6 month frozen cortex, as compared to fresh values thus suggesting that specific ^{3}H-dexamethasone binding to hippocampal CR may be altered following long periods of freezing (up to 6 months). However, the inconsistency of the observed reductions in B_{max} values (seen after 6, but not 12 months of freezing) suggested that the differences are more likely to be due to experimental error than induced by freezing. All subsequent binding experiments were conducted in as short a time as possible after collection and freezing of samples and also with control samples being assayed in parallel. A closer investigation of CR binding parameters obtained over three years showed B_{max} and K_D values to be quite stable over long periods of time. This is discussed further in Chapter 7.

2.12.7. Determination of total CR (type I and II) and type II (GR) using RU28362

There were no differences between specific ^{3}H-dexamethasone binding in the presence of fixed or variable concentrations of RU28362. For subsequent experiments in which type II CR were distinguished, a fixed concentration of RU28362 (0.5μM) was used in the assay.

The very similar values obtained for specific ^{3}H-dexamethasone binding parameters in type I + II CR and in type II CR alone (as measured using RU28362), suggested that the receptors being measured in the total CR population were predominantly of the type II variety. Type II receptor affinities for ^{3}H-dexamethasone (K_D = 2-5nM) obtained in previous studies (Reul & de Kloet, 1985; Vedder *et al*, 1993) were consistent with our data supporting the hypothesis that the receptors we measured using this assay were mainly type II corticosteroid receptors.

The competition binding studies with ^{3}H-dexamethasone have also demonstrated a glucocorticoid-like pattern of receptor binding, ie; RU28362 > DEX > ALDO (Vedder *et al*, 1993).

Investigations into the relative occupancy of type I and II CR at the time of sacrifice and dissection have shown that approximately 70-90% of type I CR are occupied under basal levels of corticosterone output. At this concentration of plasma corticosterone (1-3 μg/100mL), only approximately 10-30% of type II CR would be occupied (Reul & de Kloet, 1985; Spencer *et al*, 1990). These levels of corticosterone would be produced during the morning trough of the circadian cycle corresponding with the time at which our animals were sacrificed.

Previous studies have investigated changes in both type I and type II CR in the rat brain (Reul *et al*, 1993; 1994; Budziszweska *et al*, 1994a; 1994b). These have been made possible by adrenalectomising the animals prior to sacrifice and dissection thereby removing sources of endogenous ligand that could possibly impede specific ^{3}H-dexamethasone binding in the CR assay. In our studies, CR concentrations were assessed in animals with intact adrenals therefore circulating endogenous corticosterone was present and, due to this, it is likely that many type I CR were occupied at the time of receptor measurement.

It is very likely therefore, considering the use of animals with intact adrenals in our experiments, that even without the use of RU 28362, the measurement of total CR populations using our methodology contains mostly available type II GR.

This section has demonstrated that the binding of ^{3}H-dexamethasone to brain cytosolic fractions under the conditions in our assay is a valid method of investigating CR that fulfils the necessary criteria for receptor binding and generates reliable data pertaining to the measurement of CR binding parameters.

CHAPTER 3

THE EFFECT OF ANTIDEPRESSANT ADMINISTRATION ON RAT PLASMA CORTICOSTERONE AND CORTICOSTEROID RECEPTORS

3.1. Introduction

Normalisation of various features of the dysfunctional HPA axis seen in depression following chronic administration of antidepressants is well documented. The long-term administration of several antidepressants is found to reduce HPA axis hyperactivity (Barden *et al*, 1995; Holsboer & Barden, 1996) and plasma cortisol release (Shimoda *et al*, 1988; Reul *et al*, 1993; 1994; Delbende *et al*, 1994). Chronic exposure to various antidepressants (amitriptyline, moclobemide, imipramine, maprotiline) has been reported to increase MR and GR binding, mostly in hippocampal and hypothalamic regions of the rat brain (Reul *et al*, 1993; Reul *et al*, 1994; Budziszweska *et al*, 1994a; 1994b). The expression of GR and MR mRNA in rat brain and cell cultures is also found to be enhanced following long term administration with the antidepressants imipramine, amitriptyline, desipramine, citalopram and maprotiline (Pepin *et al*, 1989; Peiffer *et al*, 1991; Seckl & Fink, 1992; Barden, 1996; Pariante *et al*, 1997).

The elevation of CR binding and CR mRNA expression in brain regions following chronic administration of various antidepressant drugs has been proposed as an explanation of the therapeutic efficacy of these drugs in the treatment of depression. Two possibilities currently exist as mechanisms of antidepressant-induced regulation of CR;

1) many antidepressant drugs are found to inhibit the re-uptake of NA and 5HT and both of these neurotransmitter systems have been found to exert a modulatory effect on the regulation of hippocampal CR concentrations (Lowy *et al*, 1990; Mitchell *et al*, 1990; Dinan, 1996a; 1996b).
2) it has been suggested that a primary action of antidepressants could be exerted at a genomic level resulting in enhanced CR gene expression and a subsequent decrease in HPA activity (Pepin *et al*, 1992; Pariante *et al*, 1997).

All previous investigations into the effects of chronic antidepressant administration on brain CR concentrations have been carried out in animals that were adrenalectomised prior to the measurement of CR binding activity. This procedure has the effect of removing residual endogenous ligand from binding sites thus enabling the measurement of changes in (available) CR concentrations under conditions of minimal nuclear occupancy. However;

i) the procedure of adrenalectomy itself is found to interfere with the regulation of CR (Reul *et al*, 1989; Chao *et al*, 1989; Spencer *et al*, 1991; Karst *et al*, 1997).

ii) in depression, CR regulation by stress and/or antidepressant treatment occurs in the presence of the endogenous ligand.

It is essential therefore to investigate CR binding activity in the presence of endogenous ligand in laboratory animals to determine whether the up-regulation of CR observed following long-term antidepressant administration is an effect observed in the adrenally-intact animal.

The neuroanatomical specificity of these effects also supports the hypothesis of altered CR plasticity following stress and antidepressant administration. Many changes in CR concentrations are observed in the hippocampus (the location of most brain MR and some GR) and the hypothalamus, both regions that are heavily involved with CR regulation. Since many of the changes in receptor plasticity are observed in GR (which are more widely distributed), the investigation of these receptors in various other brain regions (and peripheral tissues) would also be of interest.

The aims of the present study were to investigate the effects of repeated administration, and withdrawal, of three antidepressants with differing initial mechanisms of action on various elements of the HPA system in adrenally intact animals (i.e. in the presence of endogenous ligand). The effects of the following antidepressants;

i) DMI (desmethylimipramine), a tricyclic

ii) paroxetine, an SSRI (selective 5-HT re-uptake inhibitor)

iii) venlafaxine, a SNRI (5-HT/NA reuptake inhibitor)

were studied on the following parameters in rats;

1. body and adrenal weights
2. the specific binding of ^{3}H-dexamethasone to CR in various brain regions and in the thymus gland
3. basal plasma corticosterone concentrations

3.2. Methods

3.2.1. Animals

Male Sprague-Dawley rats (200-250g; NESCOT breeding colony, U.K.) were housed 4 per cage under conditions of standard lighting (12:12 hour light-dark cycle), temperature (18-22°C) and humidity (~55%). Food and water were available *ad libitum.*

3.2.2. Antidepressant drug administration

The antidepressant drugs used in these studies were;

i) the tricyclic desmethylimipramine, DMI (Sigma, U.K.)

ii) paroxetine, a selective 5-HT reuptake inhibitor (SmithKline Beecham, U.K.)

iii) venlafaxine, a combined 5-HT/NA reuptake inhibitor (Wyeth Laboratories, U.K.).

Investigations into the effects of these drugs were carried out separately with control groups being assigned to each of the drug treatment groups. Animals were randomly assigned to a number of different groups (corresponding to the period after beginning drug administration at which the animals were sacrificed) each consisting of 8 control and 8 drug treated rats. Body weights were recorded regularly throughout the period of drug administration and withdrawal. Rats were dosed once daily p.o. (between 08.00-09.00hrs) for up to 28 days with either distilled water vehicle or desmethylimipramine (10mg/kg), paroxetine (5 mg/kg) or venlafaxine (15 mg/kg). The oral route of drug administration was chosen to ensure that each of the animals received the stated dose of antidepressant and that metabolism of the drug would occur via the gastro-intestinal route as in oral administration of antidepressants in humans.

3.2.3. Tissue collection and ^{3}H-dexamethasone binding assay

Sacrifice of rats took place between 08.00-11.00 hours in a separate room at various times 24 hrs after the last dose or following 7 days of discontinuing drug treatment. Dissection of brain regions, thymus and adrenals was carried out as described in section 2.3.2. Trunk blood was collected and the preparation of serum was carried out as described in section 3.2.4. All tissues were stored at -70°C until required for further analysis.

i) Displacement experiments - The antidepressant drugs being used in these studies were tested for displacement of ^{3}H-dexamethasone binding to cytosolic sites prior to commencement of binding studies. Competition assays were carried out as described in section 2.5 with each of the antidepressant compounds diluted in distilled water and added to the assay in concentrations ranging from $5x10^{-12}$ to $5x10^{-3}$M.

ii) Saturation experiments - Saturation binding assays were carried out as described in section 2.3. Incubations were carried out in duplicate for total binding and singly for binding in the presence of 5μM hydrocortisone and 0.5μM RU28362. Saturation experiments in cortex, hippocampus and thymus were carried out in cytosolic preparations using tissue from one animal. Saturation assays in striatal and hypothalamic regions were carried out in cytosolic preparations using tissue pooled from two animals. ^{3}H-dexamethasone binding to CR was measured at 6 ligand concentrations (0.625-20nM) in the DMI and venlafaxine studies and at 8 ligand concentrations (0.325-20nM) in the paroxetine study. Tissues from both vehicle and antidepressant drug treated animals were compared within the same assays in order to minimise the effects of daily variations in assay procedures. Protein concentrations

were determined by the method of Lowry *et al* (1951) with BSA as the standard (see section 2.11).

3.2.4. High performance liquid chromatography (HPLC) determination of plasma corticosterone concentrations

3.2.4.a Introduction

Measurements of plasma corticosteroids are commonly performed using the radioimmunoassay method. However it has been shown that the quantitative analysis of endogenous glucocorticoids using HPLC techniques can yield accurate and reproducible data (Shimizu *et al*; 1983, Hariharan *et al*; 1992). We decided to use this method for analysis of plasma corticosterone concentrations in rats treated for up to 28 days with DMI, paroxetine or venlafaxine.

3.2.4.b Methods

i) Serum extraction from blood samples

Trunk blood samples were collected at the time of sacrifice, following decapitation of the animals. Approximately 10mL of blood was collected in a 50mL centrifuge tube and stored at room temperature for 30 min following which samples were placed at 4°C for 1hour. Samples were then removed and left at room temperature for 15 mins and the resulting blood clot was dislodged by gently introducing a glass rod around the clot and the wall of the tube. The supernatant (serum) was transferred by pipette to another tube and spun at 2500g for 10 min at room temperature, transferred to a cryovial and stored at -70° until corticosterone extraction.

ii) Solid phase extraction of corticosterone

Following the thawing of serum samples, they were heated for 10 minutes at 52°C. 400µL of trichloroacetic acid was added to the serum sample resulting in a cloudy precipitate. Contents were transferred to an eppendorf tube and spun in a microfuge for 4 minutes at 5000g. 25µL of nortestosterone standard was added to the supernatant to give a final nortestosterone concentration of 2.5µg/mL as an internal standard. The sample was loaded onto a preconditioned solid phase extraction (SPE) column.

iii) Preconditioning of SPE column

Columns were prepared by rinsing with 1mL methanol (Hypersolv Grade) and allowing elution under gravity. Flushing the column with 1mL distilled water displaced the methanol following which, 500µL of corticosterone extract was loaded onto the column. The column was then washed with 1mL distilled water to wash away the soluble particles. The analyte (consisting of hydrophobic components such as corticosterone) was then eluted under gravity with methanol and the eluate collected and stored at -70°C until ready for HPLC analysis.

iv) HPLC of standards and corticosterone extract

Mobile phase (65% methanol + 35% distilled water) was filtered and degassed with helium for 20 mins prior to use. The pump was primed with mobile phase for a minimum of 20 mins before the injection of samples into the HPLC apparatus. Standards of corticosterone and nortestosterone were prepared in Hypersolv methanol to give 2.5:2.5µg/mL concentrations and loaded into the HPLC apparatus.

3.3 Calculations and analysis of results

3.3.1. Competition assays

Analysis of all competition experiments was carried out as described in section 2.5. Competition profiles are displayed in Appendix 3.4.1.

3.3.2. Saturation assays

The binding assay described in section 2.4 was used to measure total CR population and also GR concentrations (using RU28362) in various rat brain regions. Only data pertaining to total receptor population (CR) is included however, as values were found to be very similar for the two receptor populations. Also, it appears that CR being measured are comprised mostly of type II GR (as discussed in sections 2.10 and 2.12). Values for specific ^{3}H-dexamethasone binding parameters to type I and II CR in various tissues from these studies are given in Appendices 3.4.2, 3.4.3 and 3.4.4.

3.3.3. HPLC determination of plasma corticosterone concentrations

The corticosterone: nortestosterone standards injected into the HPLC were equivalent to concentrations of 0.05µg and 0.05µg respectively, these being used to calculate the response factors (Rf) and retention time (Rt) for each peak. Following the injection of the sample, the nortestosterone internal standard was determined from the Rf and Rt of the standards. Corticosterone peaks were also identified in the sample and matched against the Rf and Rt for standard corticosterone. The area of this peak was used to calculate the concentration of corticosterone in the sample. Corrections were made for the data if the recovery of the internal standard was not found to be 100%.

3.3.4. Statistical analysis

The mean (± s.e.m.) of individual determinations were calculated for each treatment group for body weights, adrenal weights, ^{3}H-dexamethasone binding parameters (B_{max} and K_D), tissue protein content and plasma corticosterone concentrations.

Normal frequency distributions and some simple correlations were determined for data before it was subjected to any other statistical procedures. Data that did not conform to a normal distribution, such as K_D and plasma corticosterone values were log-transformed prior to statistical analysis.

Data obtained in the time course experiments for all parameters were tested for statistically significant differences by two-way analysis of variance (ANOVA) followed by Student's t-test where the F-ratio was significant ($p<0.05$) using GB-STAT v6.0.

3.4. Results

3.4.1. Displacement assays using antidepressant compounds

DMI, paroxetine and venlafaxine did not compete for ^{3}H-dexamethasone binding to CR in rat cortex in a concentration dependent manner (Appendix 3.4.1).

3.4.2. DMI study

a) Body /adrenal weights

Data obtained for body and adrenal weight values conformed to a normal distribution.

Body weights in DMI treated animals generally remained lower than in vehicle treated control animals throughout the study (Table 3.4.2.A). However this effect was not statistically significant until day 21 of drug administration when a reduction of 10% in body weight of DMI treated rats was observed (as compared to vehicle treated controls). Animals receiving DMI however appeared to gain weight at the same rate as those receiving distilled water vehicle.

Adrenal weights (per 2 adrenals) in rats receiving DMI were significantly lower than in controls following 14 days (-19%) and 21 days of drug administration (-21% – Table 3.4.2.B). No difference was observed between DMI and vehicle treated groups after this time. Values obtained for adrenal weights 7 days after withdrawal of DMI were no different from values in vehicle treated animals or those obtained after 28 days of drug administration.

b) ^{3}H-dexamethasone binding to CR

B_{max} values conformed to a normal distribution however, K_D data did not and therefore was log transformed before being subjected to analysis of variance.

Cortical CR concentrations were 36% higher after 14 days of DMI administration (Figure 3.4.2.i.A, Appendix 3.4.2). There were no differences in CR numbers between vehicle and DMI treated animals at any other time. The K_D of specific ^{3}H-dexamethasone binding to CR in rat cortex was not consistently affected by DMI administration but was significantly lower (-41%) than values in vehicle treated animals at day 1 (Figure 3.4.2.i.B). There were no differences between values for CR binding parameters obtained following 7 days of DMI withdrawal as compared to control vehicle treated values or those measured at 28 days of treatment.

No significant effects on the B_{max} and K_D of specific ^{3}H-dexamethasone binding to CR in rat hippocampus were observed following the administration or withdrawal of DMI (Figure 3.4.2.ii).

c) Lowry protein assay

There were no significant differences in protein content of vehicle and DMI treated animals in either cortex or hippocampus (Appendix 3.4.2).

Simple correlation analysis showed no statistically significant relationships between B_{max} / K_D and protein values in both cortex and hippocampus.

d) Plasma corticosterone concentrations

Plasma corticosterone values were log transformed prior to being analysed using ANOVA. Repeated DMI administration had no consistent effect on basal corticosterone concentration in rats (Figure 3.4.2.iii). Concentrations of corticosterone in DMI treated animals were significantly lower than in vehicle treated controls at day 1 of drug administration (-38%). Plasma corticosterone concentrations in rats receiving DMI for more than 7 days were generally lower than in vehicle treated animals, however no statistically significant effects were observed at any other time points following DMI administration. Corticosterone concentrations in animals withdrawn from DMI for 7 days were significantly lower than in vehicle treated control animals (-73%).

Two-way ANOVA showed no significant interactions between drug treatment (DMI or distilled water) and time (1, 3, 7, 14, 21, and 28 days) for data on body weight, adrenal weight, CR binding parameters, tissue protein content or plasma corticosterone concentration in cortex or hippocampus.

Table 3.4.2.

Time course of the effect of DMI administration on body weight (A) and adrenal weight (B).

(A)

Time after administration of DMI or vehicle (days)	CONTROL body weight (g)	DMI body weight (g)
1	**310 ± 5** (n=56)	**294 ± 4** (n=56)
3	**280 ± 7** (n=48)	**297 ± 5** (n=48)
7	**332 ± 5** (n=40)	**316 ± 6** (n=40)
14	**359 ± 7** (n=32)	**329 ± 7** (n=32)
21	**384 ± 9** (n=24)	**347 ± 9** * (n=24)
28	**401 ± 17** (n=16)	**385 ± 16** (n=16)

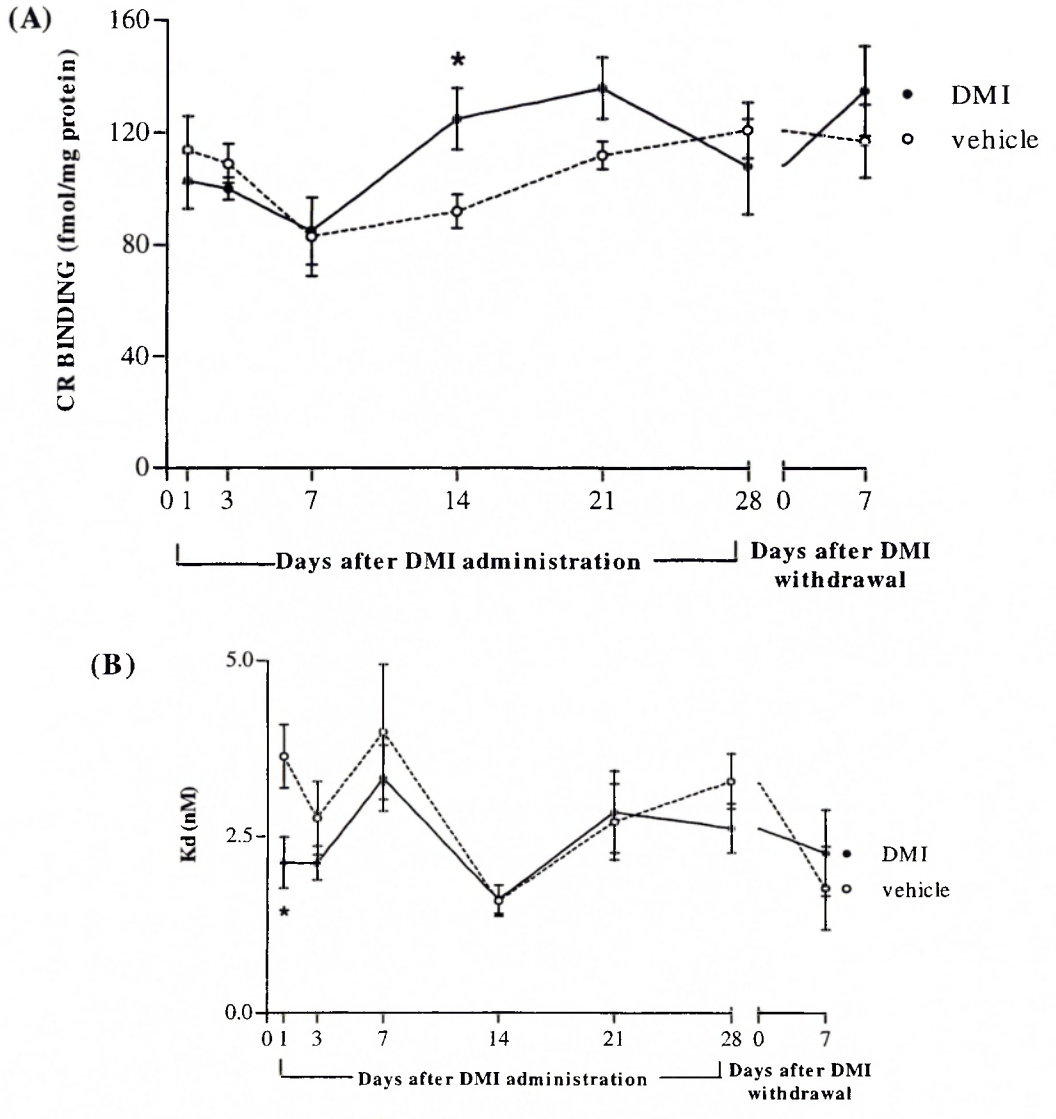
(B)

Time after administration of DMI or vehicle (days)	CONTROL adrenal weight (mg)	DMI adrenal weight (mg)
1	55 ± 4	60 ± 4
3	62 ± 4	53 ± 2
7	61 ± 5	51 ± 3
14	54 ± 3	44 ± 2 *
21	66 ± 4	52 ± 3 *
28	57 ± 5	56 ± 5
7 days after withdrawal	56 ± 2	50 ± 3

Data for adrenal weights (B) are expressed as the weight per two adrenals in mg (mean ± sem). Rats (7-8 per group) received DMI (10mg/kg p.o.) or distilled water once daily and were sacrificed after 1, 3, 7, 14, 21 and 28 days of administration and 7 days following withdrawal of the drug (see section 3.2). Statistically significant differences were determined by two-way ANOVA followed by Student's t-test where appropriate. ***ANOVA: Body weight*** *– effect of treatment F (1, 94) = 2.82, p=0.97; - effect of time F (5,94) = 18.31, p<0.0001; - interaction F (5,91) = 1.5, p=2.0.* ***Adrenal weight*** *– effect of treatment F (1, 91) = 8.78, p=0.004; - effect of time F (5, 91) = 1.77, p=0.13; - interaction F (5, 91) = 1.79, p=0.12.* ***Student's t-test; * p<0.05***

Figure 3.4.2.i

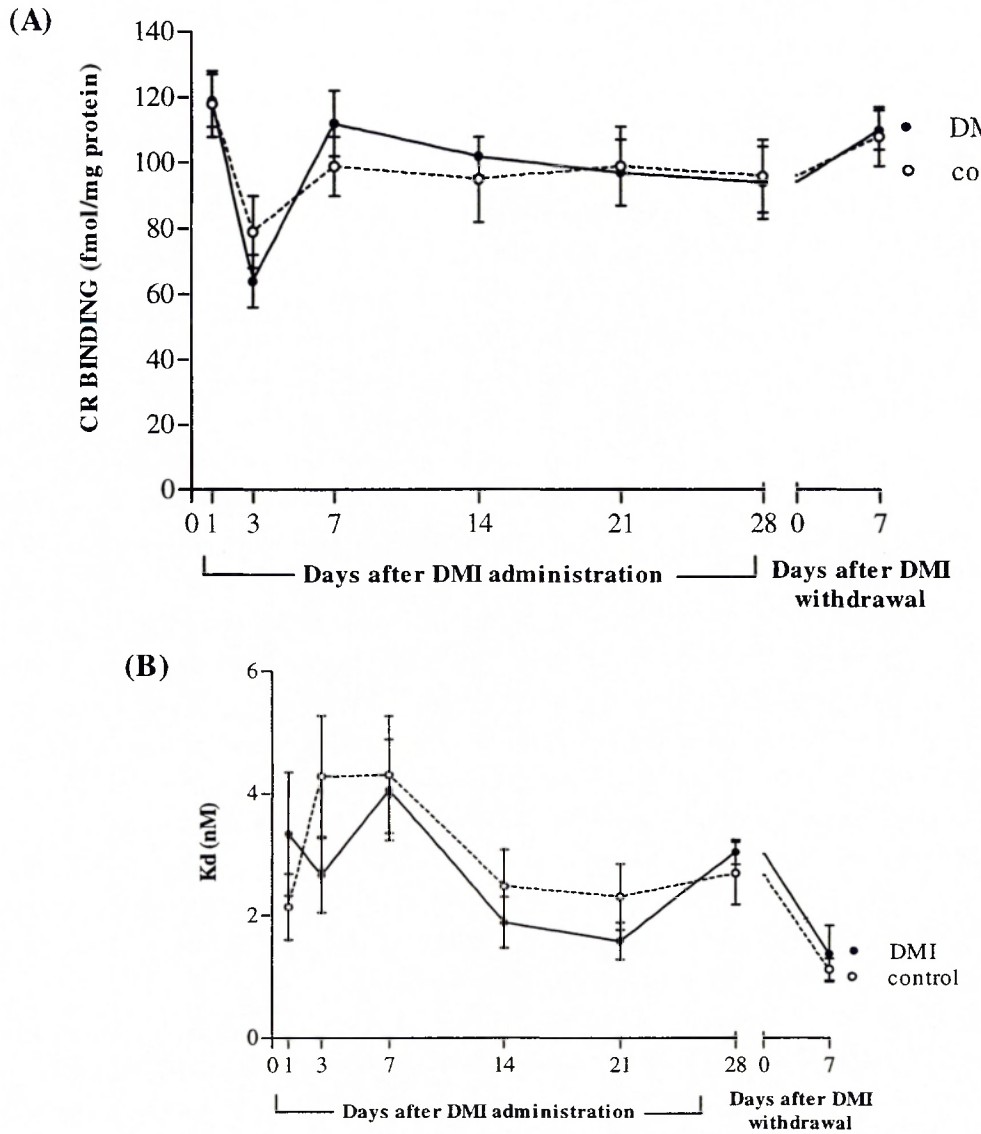
Time course of the effect of DMI administration on
B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **cortex**.



Rats (6-8 per group) received DMI (10 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 3, 7, 14, 21 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Data are expressed as mean ± sem. Mean B_{max} values ranged from 83-121 fmol/mg protein (vehicle groups) and 85–136 fmol/mg protein (DMI groups). Mean K_D values in this region ranged from 1.6-4 nM (vehicle groups) and 1.6–2.8 nM (DMI groups). Mean protein values ranged from 161-201 µg/assay (vehicle groups) and 257-207 µg/assay (DMI groups). Statistically significant differences were determined using two-way ANOVA followed by Student's t-test where appropriate. See Appendix 3.1(A). ***ANOVA: CR (B_{max})*** *-effect of treatment F (1, 88)=0.5, p=0.48; -effect of time F (5, 88)=3.44, p=0.0074; -interaction F (5, 88) =1.78, p=1.27.* ***CR (log K_D)*** *-effect of treatment F(1, 88) =1.74, p=0.19; - effect of time F (5, 88)=3.5, p=0.0067; -interaction F (5, 88)=0.96, p=0.44.* ***Student's t-test;*** **(p<0.05).*

Figure 3.4.2.ii

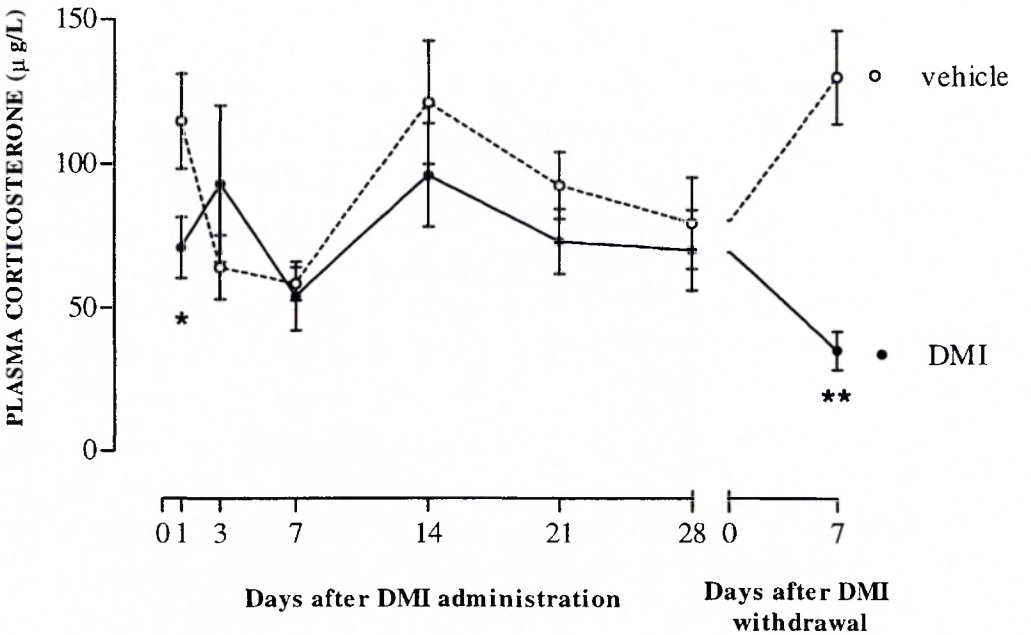
Time course of the effect of DMI administration on
B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **hippocampus**.



Rats (7-8 per group) received DMI (10 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 3, 7, 14, 21 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Data are expressed as mean ± sem Mean B_{max} values ranged from 79-118 fmol/mg protein (vehicle groups) and 64–119 fmol/mg protein (DMI groups). Mean K_D values in this region ranged from 1.1-4.3 nM (vehicle groups) and 1.6–4.1 nM (DMI groups). Mean protein values ranged from 124-171 µg/assay (vehicle groups) and 130-157 µg/assay (DMI groups). No statistically significant differences were determined using two-way analysis of variance or Student's t-tests. See Appendix 3.1.(B). ***ANOVA: CR (B_{max})*** *- effect of treatment F (1, 89) = 0.0014, p=0.97; - effect of time F (5, 89) = 4.65, p=0.0009; - interaction F (5, 89) = 4.65, p=0.82.* ***CR (log K_D)*** *- effect of treatment F (1, 89) = 0.10, p=0.75; - effect of time F (5, 89) = 3.1, p=0.013; - interaction F (5, 89) = 0.84, p=0.52*

Figure 3.4.2.iii

Time course of the effect of DMI administration on rat plasma corticosterone concentrations.



Rats (7-8 per group) received DMI (10 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 3, 7, 14, 21 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Mean plasma corticosterone concentrations ranged from 58 – 129 µg/L (vehicle groups) and 54 - 96 (DMI groups). Data is expressed as mean ± sem. Statistically significant differences were determined using two-way ANOVA followed by Student's t-test where appropriate. ***ANOVA: log plasma [corticosterone]*** *- effect of treatment F (1, 94) = 2.98, p=0.088; - effect of time F (5, 94) = 2.86, p= 0.02; - interaction F (5, 94) = 0.80, p=0.55.* ***Student's t-test;*** ** (p<0.05) or ** (p< 0.0001).*

3.4.3. Paroxetine study

a) Body / adrenal weights

Data obtained for body and adrenal weight values conformed to a normal distribution curve. There were no significant effects of paroxetine administration or withdrawal on body weight (Table 3.4.3.A). No difference was observed in adrenal weights between animals receiving paroxetine and distilled water vehicle at 1 and 28 days of drug administration (Table 3.4.3.B).

b) ^{3}H-dexamethasone binding to CR

B_{max} data conformed to a normal distribution, however K_D values did not and therefore were log transformed before being subjected to analysis of variance. CR concentrations in rat cortex were significantly lower (-33%) than control values following 14 days of paroxetine administration but were not different from vehicle treated controls at any other time points (Figure 3.4.3.i.A, Appendix 3.4.3). There was no effect of paroxetine on the K_D of specific ^{3}H-dexamethasone binding to CR in rat cortex (Figure 3.4.3.i.B).

No consistent, statistically significant differences in hippocampal B_{max} values were observed at any time following the administration of paroxetine (Figure 3.4.3.ii.A.).. The K_D of ^{3}H-dexamethasone binding to CR at day 28 of paroxetine administration was significantly lower than in the control group (-41% - Figure 3.4.3.ii.B.). Paroxetine withdrawal did not appear to affect B_{max} or K_D values in hippocampus.

CR concentrations in rat striatum were significantly higher (+32%) than vehicle treated control values following 7 days of paroxetine administration but were not different from control values at any other time points (Figure 3.4.3.iii.A). Paroxetine withdrawal had no significant effects on B_{max} values in the striatum. There were no significant

effects of paroxetine administration or withdrawal on the K_D of specific ^{3}H-dexamethasone binding to CR in rat striatum (Figure 3.4.3.iii.B).

The B_{max} of specific ^{3}H-dexamethasone binding to CR in the thymus was significantly reduced (-24%) following 28 days of paroxetine administration (Figure 3.4.3.iv.A). K_D values were not altered in rat thymus following drug administration or withdrawal (Figure 3.4.3.iv.B).

d) Lowry protein assay

There were no significant differences in protein content between vehicle and paroxetine treated animals, in any of the tissues investigated (Appendix 3.4.3).

Simple correlation analysis showed no statistically significant relationships between B_{max} / K_D and protein content ($p>0.05$) in either cortex or hippocampus in this study.

e) Plasma corticosterone concentrations

Plasma corticosterone values were log transformed and fitted to a normal distribution curve before being subjected to analysis of variance. Corticosterone concentrations were significantly elevated in animals receiving paroxetine for 14 days (+31% - Figure 3.4.3.v). There was a significant reduction in plasma corticosterone concentrations following paroxetine withdrawal (-44%) as compared to values in vehicle treated animals.

No significant interactions were observed between drug treatment (paroxetine or distilled water) and time (1, 7, 14 and 28 days) for data on body weight, adrenal weight,

CR binding parameters / protein content in cortex, hippocampus, striatum and thymus or plasma corticosterone concentrations using two-way ANOVA.

Table 3.4.3.

Time course of the effect of paroxetine administration on body weight (A) and adrenal weight (B).

(A)

Time after administration of PAROXETINE or vehicle (days)	CONTROL body weight (g)	PAROXETINE body weight (g)
1	254 ± 2 (n=40)	246 ± 3 (n=40)
7	293 ± 3 (n=32)	290 ± 4 (n=32)
14	321 ± 5 (n=24)	319 ± 6 (n=24)
28	356 ± 4 (n=16)	365 ± 9 (n=16)
7 days after withdrawal	359 ± 6 (n=8)	368 ± 15 (n=8)

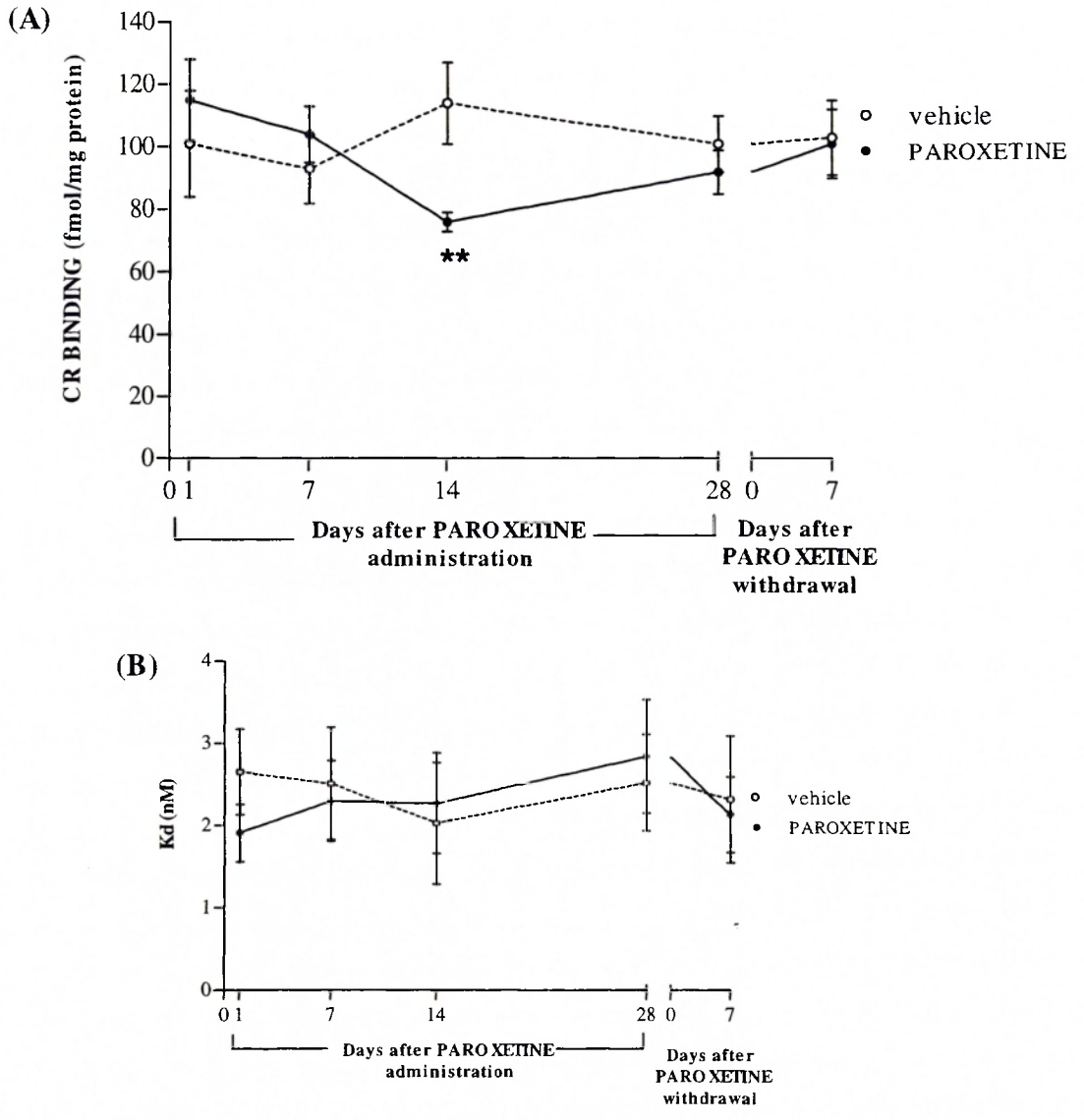
(B)

Time after administration of PAROXETINE or vehicle (days)	CONTROL Adrenal weight (g)	PAROXETINE Adrenal weight (g)
1	45 ± 2	45 ± 3
7	-	-
14	-	-
28	59 ± 3	57 ± 3
7 days after withdrawal	-	-

Data for adrenal weights (B) are expressed as the weight per two adrenals in mg (mean ± sem). Rats (8 per group) received paroxetine (5mg/kg p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). No statistically significant differences were determined using two-way ANOVA or Student's t-tests. *ANOVA: Body weight – effect of treatment $F(1, 63) = 1.35$, $p=0.25$; - effect of time $F(3,63) = 28.25$, $p<0.0001$; - interaction $F(3, 56) = 0.81$, $p=0.49$. Adrenal weight – effect of treatment $F(1, 31) = 0.02$, $p=0.89$; - effect of time $F(1, 31) = 24.43$, $p<0.0001$; - interaction $F(1, 31) = 0.23$, $p=0.63$.*

Figure 3.4.3.i

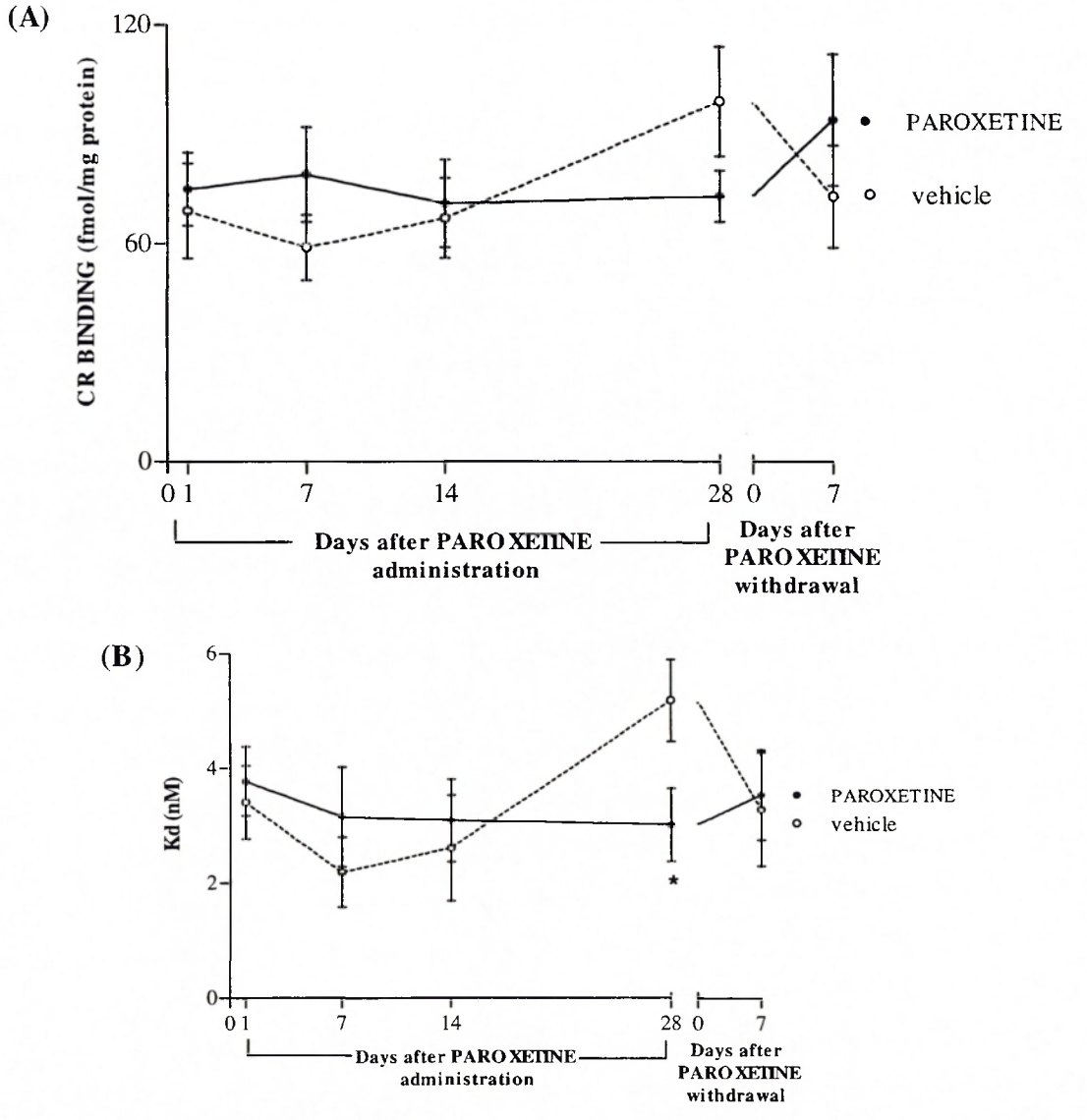
Time course of the effect of paroxetine administration on B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **cortex.**



Rats (7-8 per group) received paroxetine (5 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (see section 3.2). Data are expressed as mean ± sem. Mean B_{max} values ranged from 93-114 fmol/mg protein (vehicle groups) and 76–115 fmol/mg protein (paroxetine groups). Mean K_D values in this region ranged from 2.3-2.7 nM (vehicle groups) and 1.9–2.8 nM (paroxetine groups). Mean protein values ranged from 145-160 µg/assay (vehicle groups) and 150-173 µg/assay (paroxetine groups). Statistically significant differences were determined using two-way ANOVA followed by Student's t-test where appropriate. See Appendix 3.2(A). ***ANOVA:(CR B_{max})-effect of treatment F(1, 58)=0.6, p=0.44; -effect of time F(3, 58)=0.57, p=0.64; interaction F(3, 58)=2.32, p=0.087. CR (log K_D) -effect of treatment F(1, 57)=1.21, p=0.28; -effect of time F(3, 57)=0.49, p=0.69; -interaction F(3, 58)=0.37, p=0.78. Student's t-test; **(p<0.01).***

Figure 3.4.3.ii

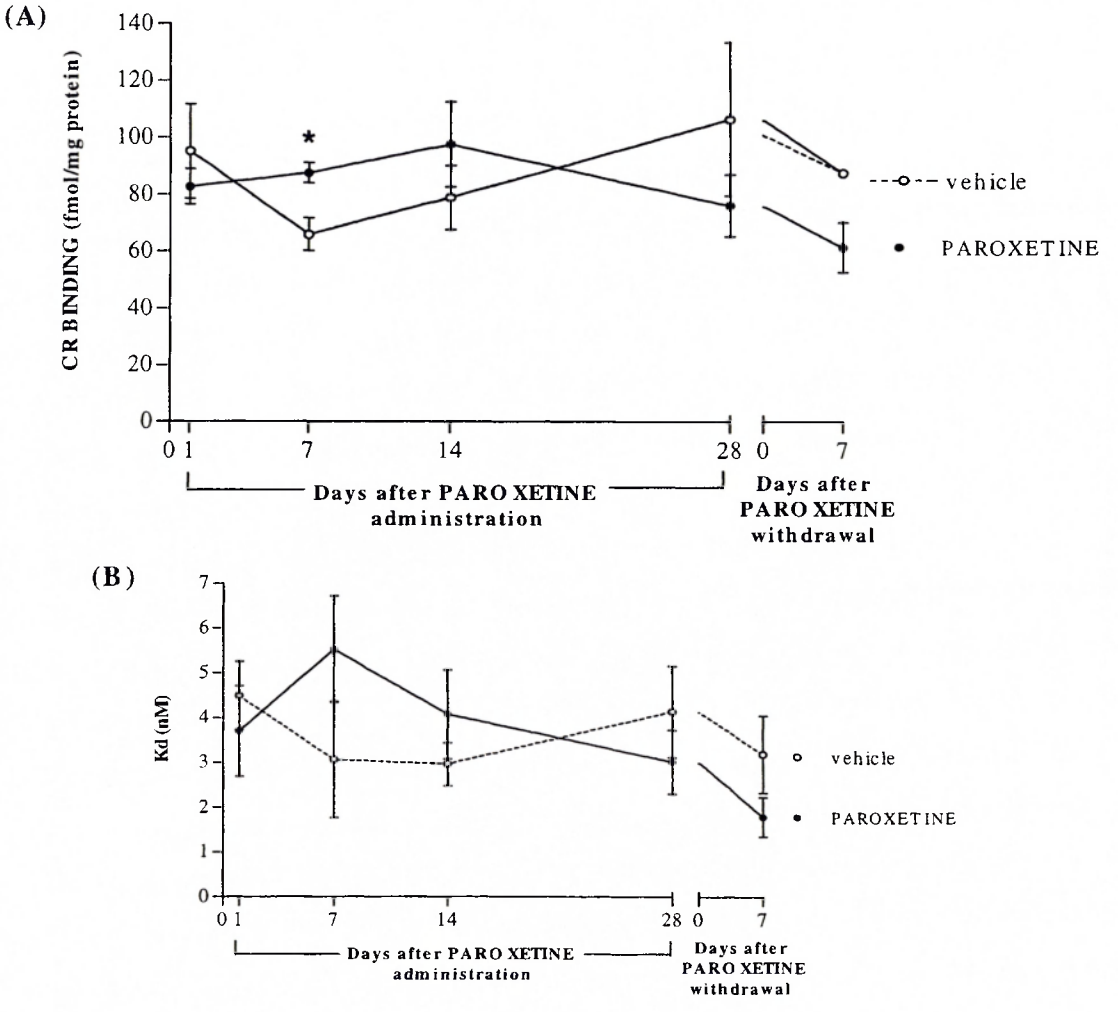
Time course of the effect of paroxetine administration on B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **hippocampus.**



Rats (6-8 per group) received paroxetine (5 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (see section 3.2). Data are expressed as mean ± sem. Mean B_{max} values ranged from 59-99 fmol/mg protein (vehicle groups) and 71–94 fmol/mg protein (paroxetine groups). Mean K_D values in this region ranged from 2.4-3.4 nM (vehicle groups) and 3–3.8 nM (paroxetine groups). Mean protein values ranged from 147-160 µg/assay (vehicle groups) and 142-156 µg/assay (paroxetine groups). Statistically significant differences were determined using two-way analysis of variance and Student's t-test where appropriate. See Appendix 3.2(B). ***ANOVA: CR (B_{max})****-effect of treament F (1, 58)=0.031, p=0.86; -effect of time F (3, 58)=1.0, p=0.4; -interaction F (3, 58)=1.44, p=0.24.* ***CR (log K_D)*** *-effect of treatment f (1, 58)=0.003 p=0.96; -effect of timeF (3, 58)=2.12, p=0.11; -interaction F (3, 58)=1.66, p=1.9.* ***Student's t-test****; *(p<0.05).*

Figure 3.4.3.iii.

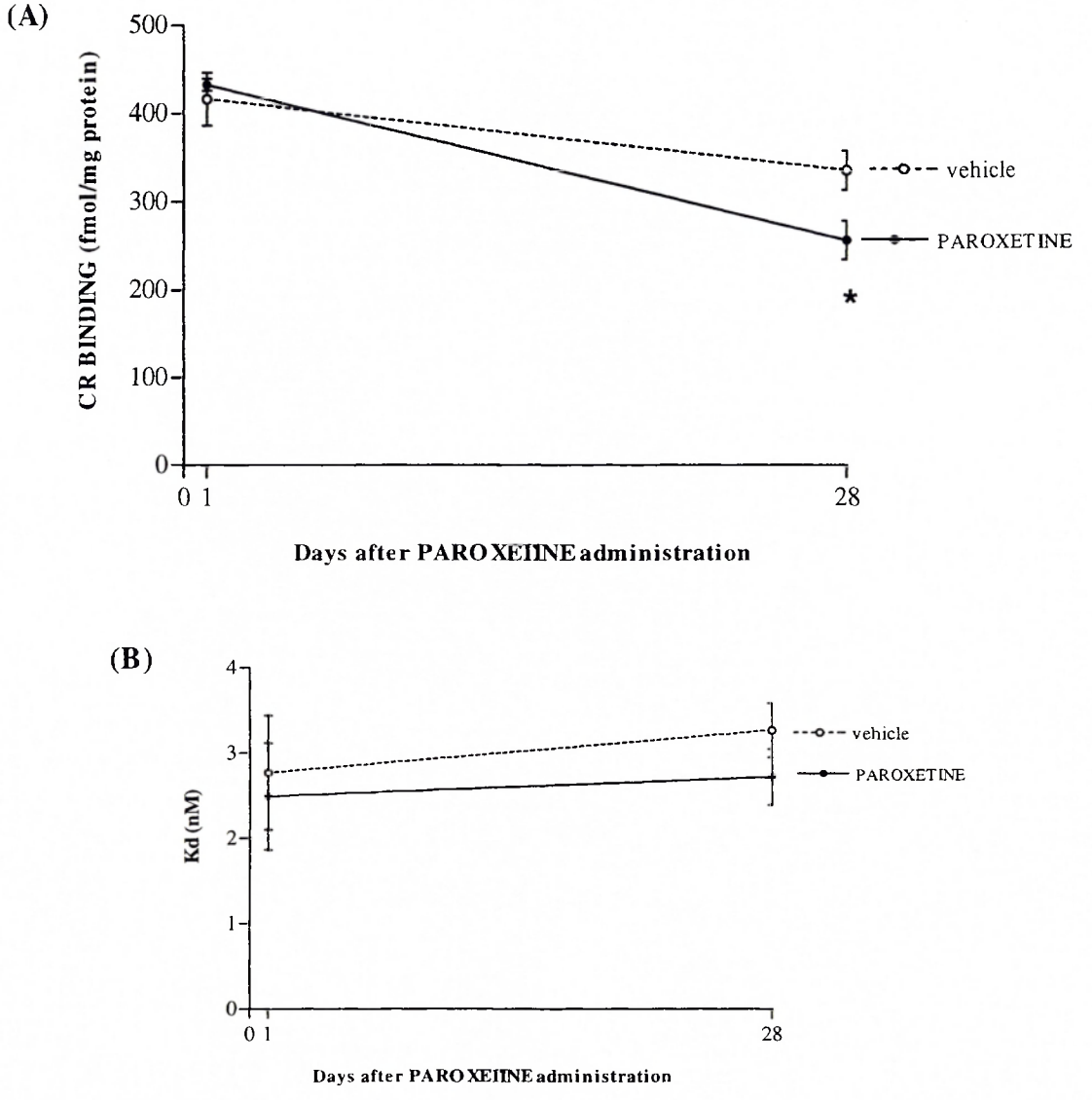
Time course of the effect of paroxetine administration on B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **striatum**.



Rats (3-4 per group) received paroxetine (5 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Striatal tissue was pooled from two animals in order to reliably conduct the CR binding assay therefore n=3-4 in each group. Data are expressed as mean ± sem. Mean B_{max} values ranged from 66-107 fmol/mg protein (vehicle groups) and 61-98 fmol/mg protein (paroxetine groups). Mean K_D values in this region ranged from 3.1-4.5 nM (vehicle groups) and 3-5.5 nM (paroxetine groups). Mean protein values ranged from 135-148 µg/assay (vehicle groups) and 132-153 µg/assay (paroxetine groups). Statistically significant differences were determined using two-way analysis of variance followed by Student's t-test where appropriate. See Appendix 3.2(C). ***ANOVA: CR (B_{max})*** *-effect of treatment F (1,28)=0.002, p=0.97; -effect of time F (3, 28)=0.44, p=0.73; -interaction (3, 28)=1.56, p=0.23.* ***CR (log K_D)*** *-effect of treatment F (1, 28)=0.51, p=0.48; -effect of time F (3, 28)=0.21, p=0.89; - interaction F (3, 28) = 1.87, p=0.17.* ***Student's t-test****; (p<0.05).*

Figure 3.4.3.iv.

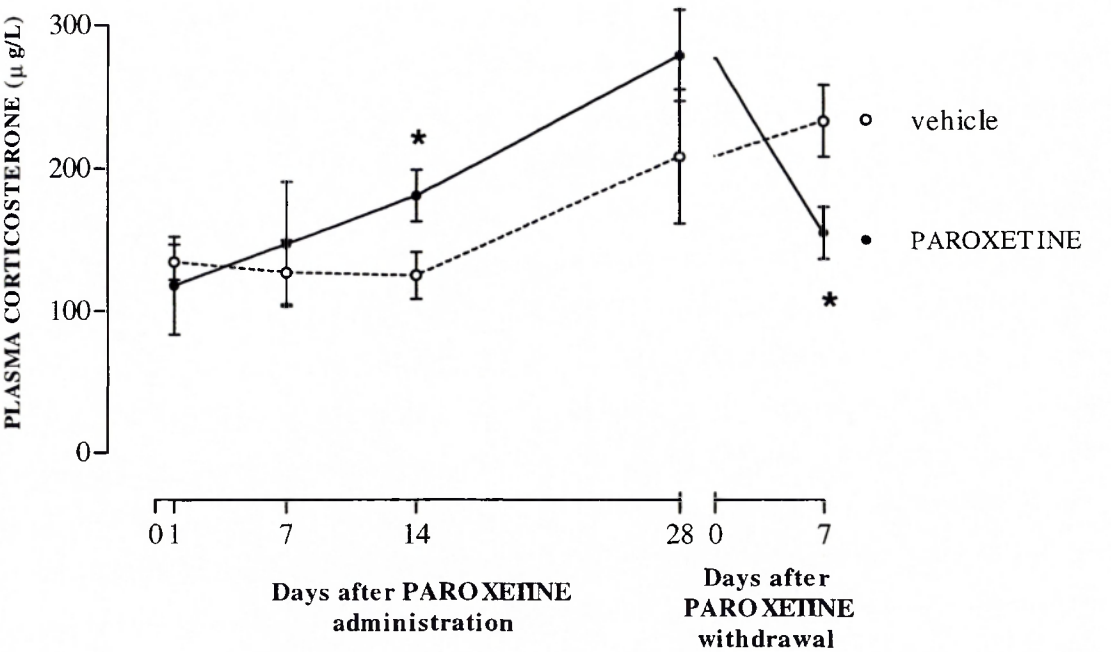
The effect of paroxetine administration on
$\mathbf{B_{max}}$ (A) and $\mathbf{K_D}$ (B) of specific ^{3}H-dexamethasone binding to CR in rat **thymus**.



Rats (7-8 per group) received paroxetine (5 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1 and 28 days of drug (as described in section 3.2). Data are expressed as mean ± sem. Mean B_{max} values ranged from 336-417 fmol/mg protein (vehicle groups) and 257–433 fmol/mg protein (paroxetine groups). Mean K_D values in this region ranged from 2.8-3.3 nM (vehicle groups) and 2.5–2.7 nM (paroxetine groups). Mean protein values ranged from 156-160 µg/assay (vehicle groups) and 152-163 µg/assay (paroxetine groups). Statistically significant differences were determined using two-way analysis of variance followed by Student's t-test where appropriate. See Appendix 3.2(D). ***ANOVA: CR*** *(B_{max}) -effect of treatment F (1, 30)=1.39, p=0.25; -effect of time F (1, 30)=22.96, p<0.0001; -interaction F (3, 30)=3.17, p=0.09. CR (log K_D) -effect of treatment F (1, 30)=0.83, p=0.37; -effect of time F (1, 30)=2.22, p=0.15; -interaction F (3, 30)=0.07, p=0.79. Student's t-test; * (p<0.05).*

Figure 3.4.3.v.

Time course of the effect of paroxetine administration on rat plasma corticosterone concentrations.



Rats (6-8 per group) received paroxetine (5 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Data are expressed as mean ± sem. Mean corticosterone concentrations ranged from 125 - 233 µg/L (vehicle groups) and 118 - 279 µg/L (paroxetine groups). Statistically significant differences were determined using two-way analysis of variance followed using Student's t-test where appropriate. ***ANOVA: log plasma [corticosterone]*** *- effect of treatment F (1, 59) = 0.68, p=0.41; - effect of time F (3, 59) = 4.19, p=0.01; - interaction F (3, 59) = 1.73, p=0.17.* ***Student's t-test;*** ** (p<0.05).*

3.4.4. Venlafaxine study

a) Body / adrenal weights

Data obtained for body and adrenal weight values conformed to a normal distribution.

No differences in body weight were observed following the administration of venlafaxine (15 mg/kg) for 28 days or 7 days of withdrawal from the drug (Table 3.4.4.A.).

There were no significant effects of venlafaxine administration for 1,14 and 28 days, on adrenal weights (Table 3.4.4.B.).

b) ^{3}H-dexamethasone binding to CR

B_{max} values conformed to normal distributions however K_D data did not and were log transformed before being subjected to analysis of variance. No statistically significant differences were observed in B_{max} values of venlafaxine treated animals as compared to vehicle treated groups (Figure 3.4.4.i.A, Appendix 3.4.4). The K_D of specific ^{3}H-dexamethasone binding to CR in rat cortex was not consistently altered by venlafaxine (Figure 3.4.4.i.B) but was significantly increased following 14 days of venlafaxine administration (+ 53%). Withdrawal of venlafaxine had no effect on B_{max} or K_D values as compared to vehicle treated controls.

Hippocampal CR concentrations were not significantly affected by venlafaxine administration (Figure 3.4.4.ii.A) and no differences were observed between values from vehicle or venlafaxine treated animals at any time points. The K_D of specific ^{3}H-dexamethasone binding to CR was not consistently altered in rat hippocampus following venlafaxine (Figure 3.4.4.ii.B) but an increase was observed following 14 days of venlafaxine administration (+45%). Withdrawal from venlafaxine did not

appear to have any effects on binding parameters of ^{3}H-dexamethasone in hippocampus.

There were no significant effects of venlafaxine administration or withdrawal on striatal CR concentrations (Figure 3.4.4.iii.A). The K_D of specific ^{3}H-dexamethasone binding to CR in rat striatum was not consistently affected by venlafaxine administration (Figure 3.4.4.iii.B) but significantly reduced after 7 days of withdrawal from the drug.

No changes in the B_{max} or K_D of specific ^{3}H-dexamethasone binding to CR were observed in rat thymus following venlafaxine administration for 1, 14 and 28 days (Figure 3.4.4.iv).

B_{max} values in hypothalamus were not significantly altered by the administration of venlafaxine over 28 days. Following the withdrawal of venlafaxine, B_{max} values in treated animals were significantly higher (+41%) than in controls. There were no significant effects of venlafaxine administration or withdrawal on the K_D of specific ^{3}H-dexamethasone binding to CR in rat hypothalamus (Figure 3.4.4.v).

c) Lowry protein assay

There were no significant differences in the protein content ($p>0.05$) of vehicle and venlafaxine treated animals in the various regions investigated (Appendix 3.4.4).

Simple correlation analysis showed no statistically significant relationships between B_{max} / K_D and protein content in the cortex and hippocampus in this study.

d) Plasma corticosterone concentrations

Values for corticosterone concentrations were log transformed prior to being subjected to analysis of variance. There were no statistically significant differences between plasma corticosterone concentrations observed in vehicle and drug treated animals following administration or withdrawal of venlafaxine (Figure 3.4.4.vi).

Two-way ANOVA showed no significant interactions between drug treatment (venlafaxine or distilled water) and time (1, 7, 14 and 28 days) for data on body weight, adrenal weight, CR binding parameters / tissue protein content in cortex, hippocampus, striatum and thymus or plasma corticosterone concentrations.

Table 3.4.4.

Time course of the effect of venlafaxine administration on body weight (A) and adrenal weight (B).

(A)

Time after administration of VENLAFAXINE or vehicle (days)	CONTROL body weight (g)	VENLAFAXINE body weight (g)
1	**274 ± 3** (n= 40)	**280 ± 4** (n= 40)
7	**323 ± 5** (n= 32)	**329 ± 5** (n= 32)
14	**361 ± 7** (n= 24)	**369 ± 7** (n= 24)
28	**428 ± 11** (n= 16)	**444 ± 13** (n= 16)
7 days after withdrawal	**438 ± 10** (n= 8)	**471 ± 21** (n= 8)

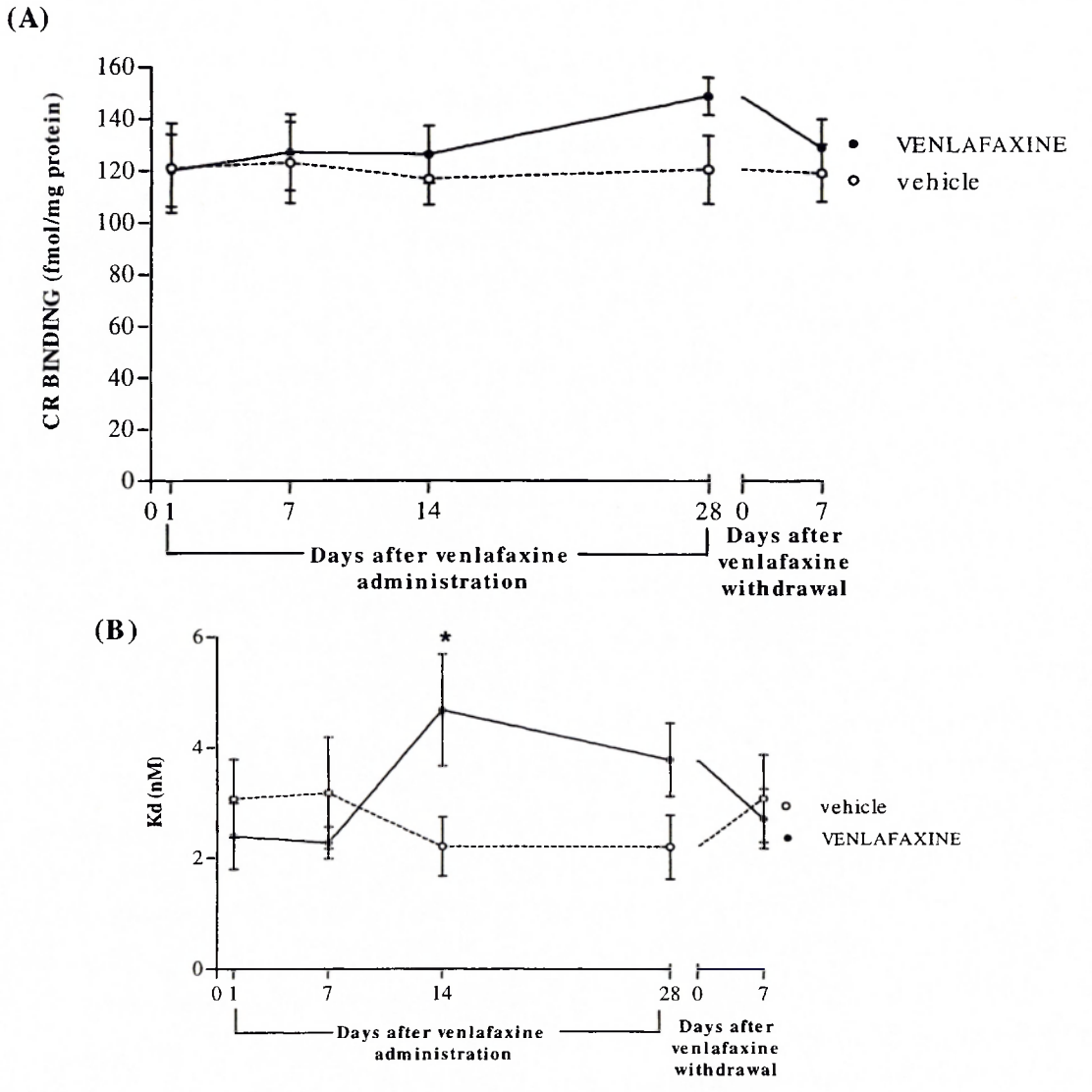
(B)

Time after administration of VENLAFAXINE or vehicle days)	CONTROL Adrenal weight (g)	VENLAFAXINE Adrenal weight (g)
1	54 ± 2	47 ± 5
7	-	-
14	47 ± 4	53 ± 3
28	49 ± 3	48 ± 3
7 days after withdrawal	-	-

Data for adrenal weights (B) are expressed as the weight per two adrenals in mg (mean ± sem). Rats (8 per group) received venlafaxine (15mg/kg p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described I section 3.2). No statistically significant differences were determined using two-way ANOVA. *ANOVA: **Body weight** – effect of treatment F (1, 63) = 0.15, p=0.70; - effect of time F (3,63) = 50.9, p<0.0001; - interaction F (3, 63) = 1.58, p=0.92. **Adrenal weight** – effect of treatment F (1, 47) = 0.01, p=0.91; - effect of time F (2, 47) = 0.24, p=0.79; - interaction F (2, 47) = 1.94, p=0.16.*

Figure 3.4.4.i

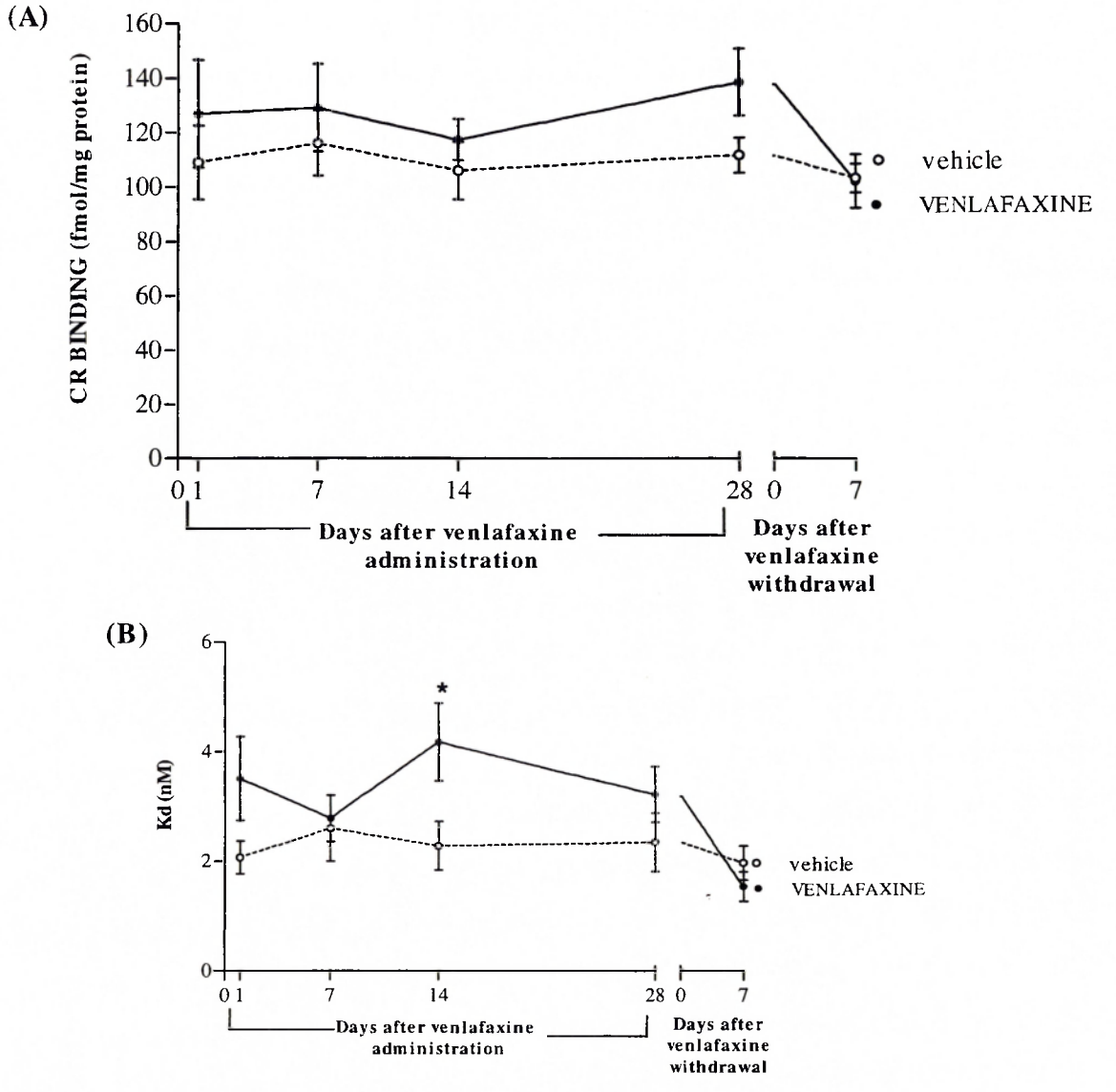
Time course of the effect of venlafaxine administration on B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **cortex**.



Rats (7-8 per group) received venlafaxine (15 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Data were expressed as mean ± sem. Mean B_{max} values ranged from 116-123 fmol/mg protein (vehicle groups) and 120–149 fmol/mg protein (venlafaxine groups). Mean K_D values in this region ranged from 2.2-3.2 nM (vehicle groups) and 2.3–4.7 nM (venlafaxine groups). Mean protein values ranged from 140-157 µg/assay (vehicle groups) and 142-158 µg/assay (venlafaxine groups). Statistically significant differences were determined using two-way ANOVA followed by Student's t-test where appropriate. See Appendix 3.3(A). ***ANOVA: CR (B_{max})*** *- effect of treatment F (1, 62)=1.18, p=0.28; -effect of time F (3, 62)=0.47, p=0.71; -interaction F (3, 55)=0.47, p=0.71.* ***CR (log K_D)*** *-effect of treatment F (1, 62)=2.96, p=0.091; -effect of time F (3, 62)=0.52, p=0.67; -interaction F (3, 62)=1.59, p=0.20.* ***Student's t-test****; * (p<0.05).*

Figure 3.4.4.ii.

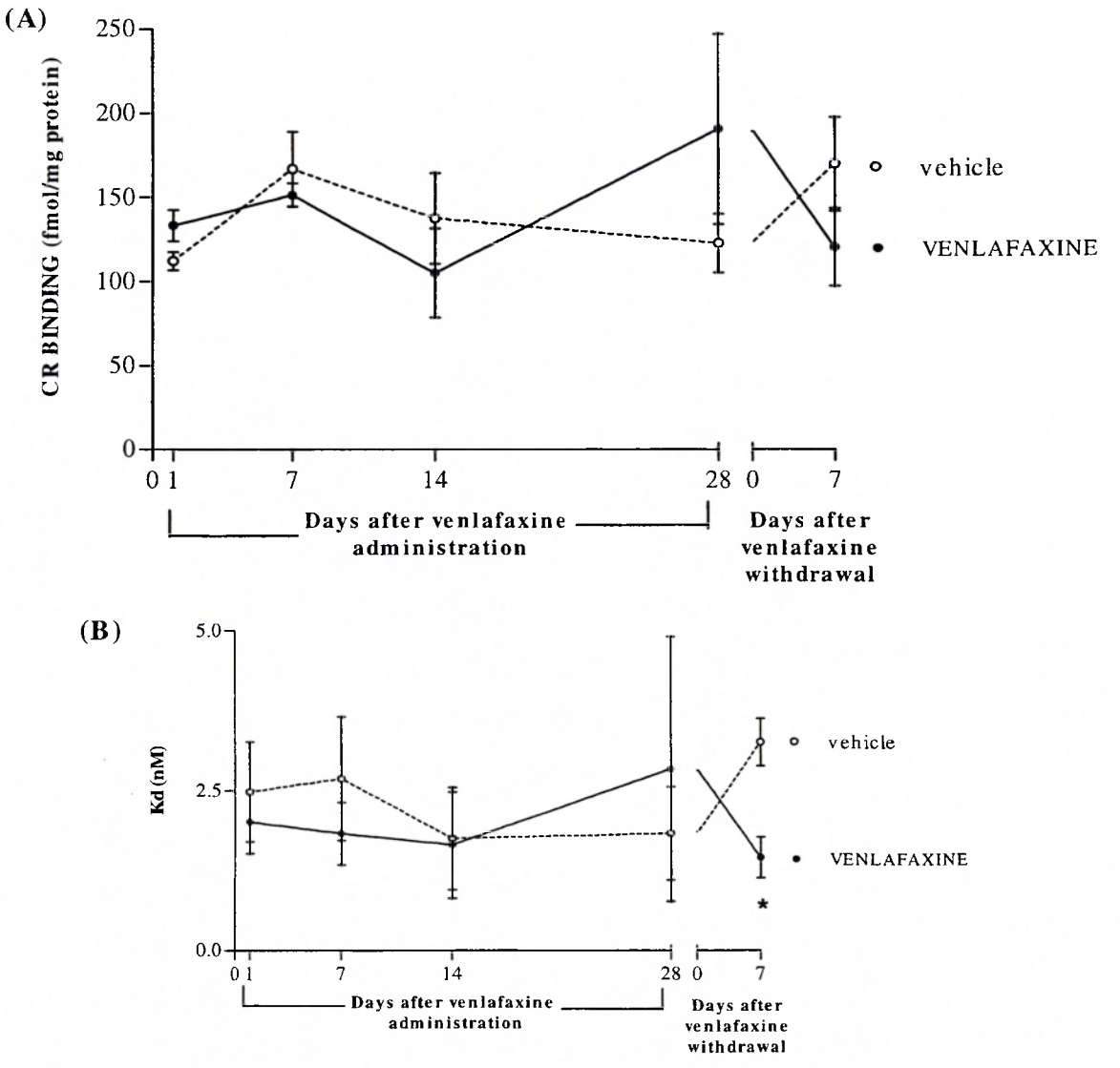
Time course of the effect of venlafaxine administration on B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **hippocampus**.



Rats (7-8 per group) received venlafaxine (15 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Data were expressed as mean ± sem. Mean B_{max} values ranged from 106-112 fmol/mg protein (vehicle groups) and 102–139 fmol/mg protein (venlafaxine groups). Mean K_D values in this region ranged from 2.1-2.6 nM (vehicle groups) and 1.5–4.2 nM (venlafaxine groups). Mean protein values ranged from 108-123 µg/assay (vehicle groups) and 108-119 µg/assay (venlafaxine groups). Statistically significant differences were determined using two-way ANOVA followed by Student's t-test where appropriate. See Appendix 3.3(B). ***ANOVA: CR (B_{max})*** *- effect of treatment F (1, 61)=3.79, p=0.57; -effect of time F (3, 61)=0.44, p=0.72; -interaction F (3, 61)=0.15, p=0.93.* ***CR (log K_D)*** *-effect of treatment F (1, 61)=7.37, p=0.009; -effect of time F (3, 61)=0.27, p=0.85; -interaction F (3, 61)=0.36,p=0.78.**Student'st-test**;*(p<0.05).*

Figure 3.4.4.iii.

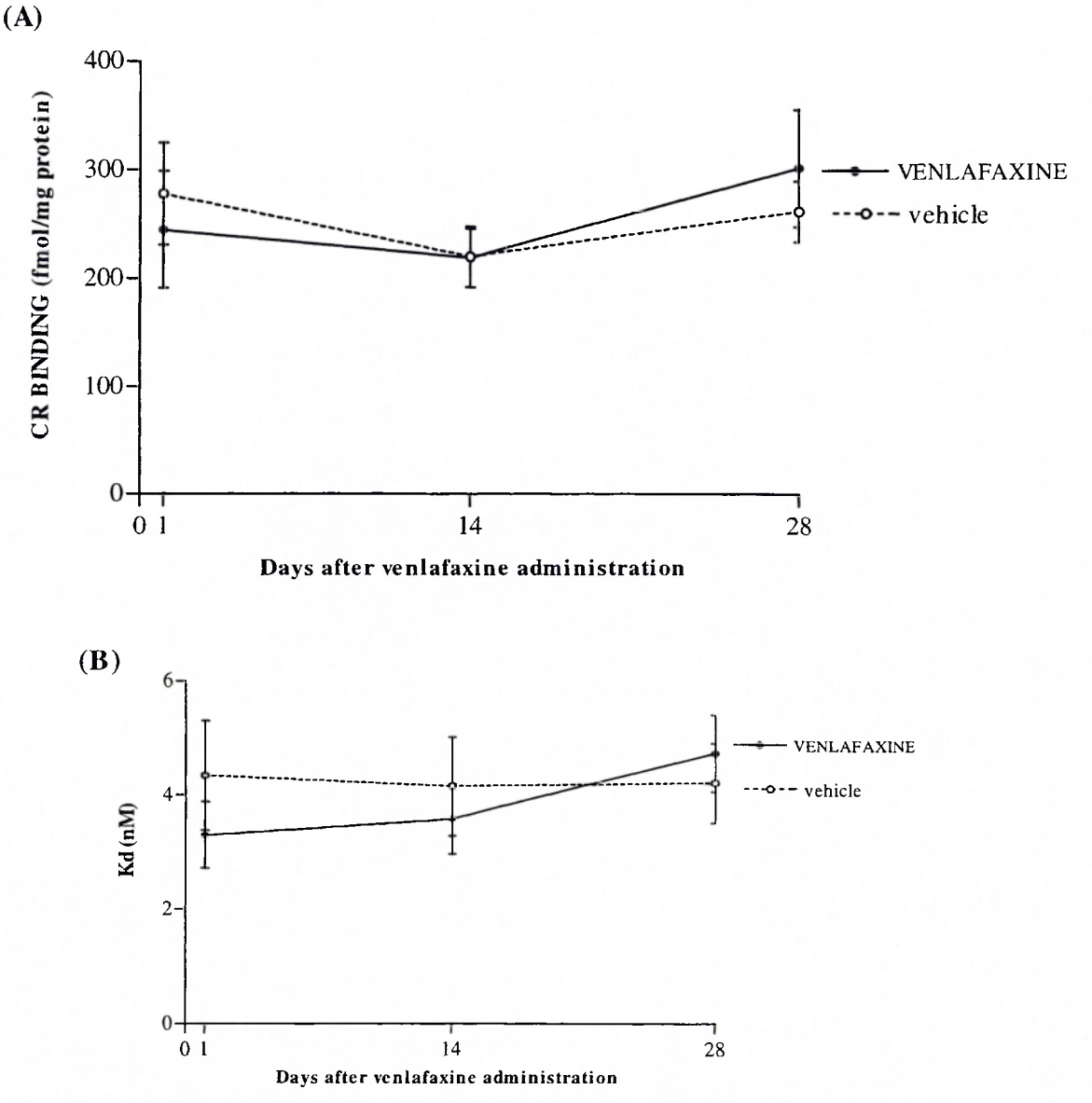
Time course of the effect of venlafaxine administration on B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **striatum**.



Rats (4 per group) received venlafaxine (15 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Striatal tissues were pooled from two animals in order to reliably conduct the CR binding assay therefore n=3-4 in each group. Data were expressed as mean ± sem. Mean B_{max} values ranged from 112-170 fmol/mg protein (vehicle groups) and 105–191 fmol/mg protein (venlafaxine groups). Mean K_D values in this region ranged from 1.8-3.3 nM (vehicle groups) and 1.7–2.8 nM (venlafaxine groups). Mean protein values ranged from 122-146 µg/assay (vehicle groups) and 130-145 µg/assay (venlafaxine groups). No statistically significant differences were determined using two-way analysis of variance or Student's t-test. See Appendix 3.3(C). ***ANOVA: CR (B_{max})*** *- effect of treatment F (1, 28)=0.33, p=0.57; -effect of time F (3, 28)=1.17, p=0.35; -interaction F (3, 28)=0.76, p=0.53.* ***CR (log K_D)*** *-effect of treatment F (1, 28)=0.058, p=0.81; -effect of time F (3, 28)=0.44, p=0.73; -interaction F (3, 28)=0.14, p=0.94.* ***Student's t-test****; * (p<0.05).*

Figure 3.4.4.iv.

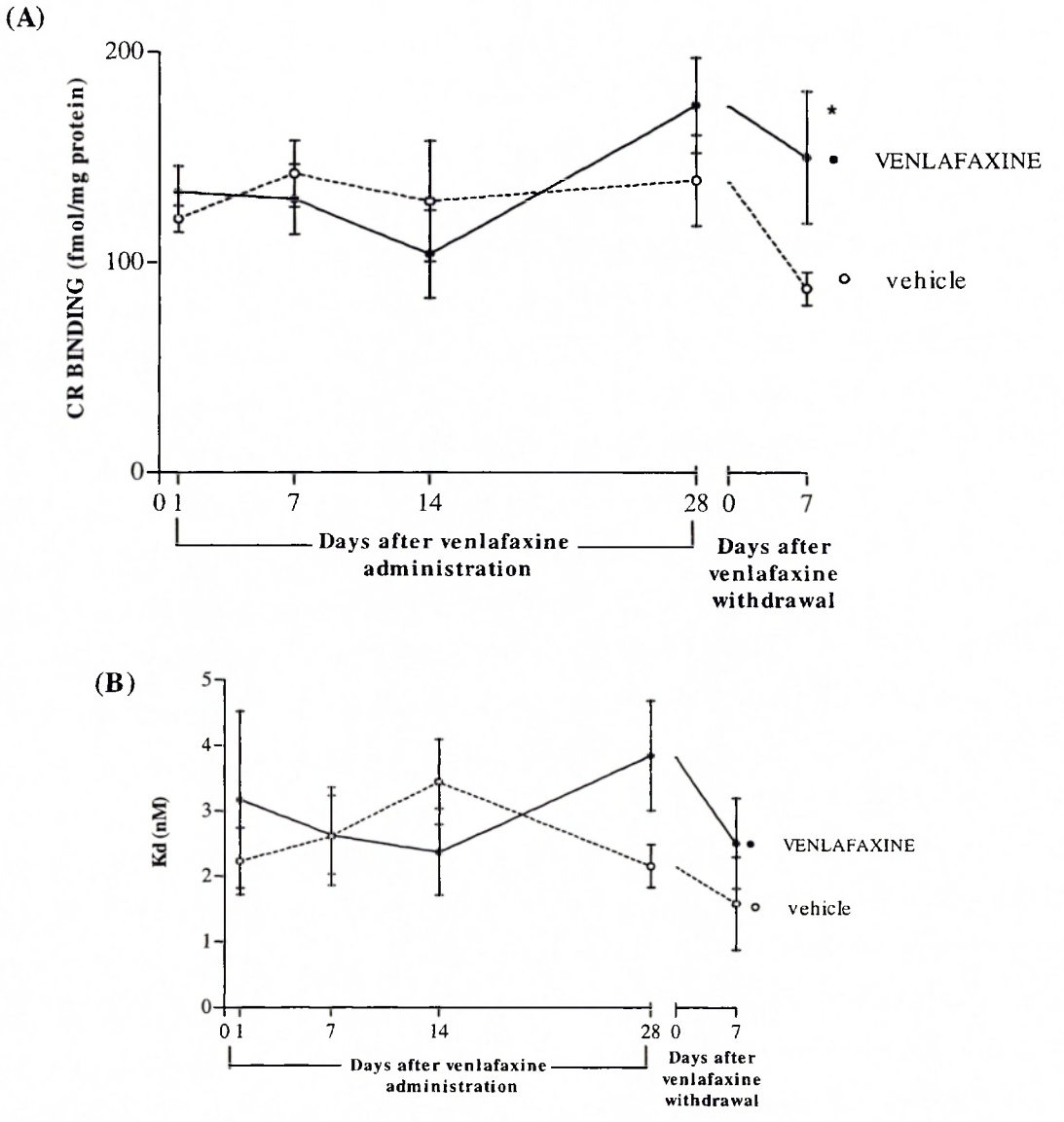
Time course of the effect of venlafaxine administration on $\mathbf{B_{max}}$ (A) and $\mathbf{K_D}$ (B) of specific ^{3}H-dexamethasone binding to CR in rat **thymus**.



Rats (7-8 per group) received venlafaxine (15 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Data were expressed as mean ± sem. Mean B_{max} values ranged from 220-278 fmol/mg protein (vehicle groups) and 219–302 fmol/mg protein (venlafaxine groups). Mean K_D values in this region ranged from 4.2-4.3 nM (vehicle groups) and 2.6–4.7 nM (venlafaxine groups). Mean protein values ranged from 111-116 µg/assay (vehicle groups) and 102-111 µg/assay (venlafaxine groups). No statistically significant differences were determined using two-way analysis of variance or Student's t-test. See Appendix 3.3(D). ***ANOVA: CR (B_{max})*** *-effect of treatment F (1, 46)=0.004, p=0.95; effect of time F (2, 46)=1.04, p=0.37; -interaction F (2, 46)=0.34, p=0.71.* ***CR (log K_D)*** *-effect of treatment F (1, 46)=0.18, p=0.68; -effect of time F (2, 46)=0.59, p=0.56; interaction F (2, 46)=0.63, p=0.54.*

Figure 3.4.4.v.

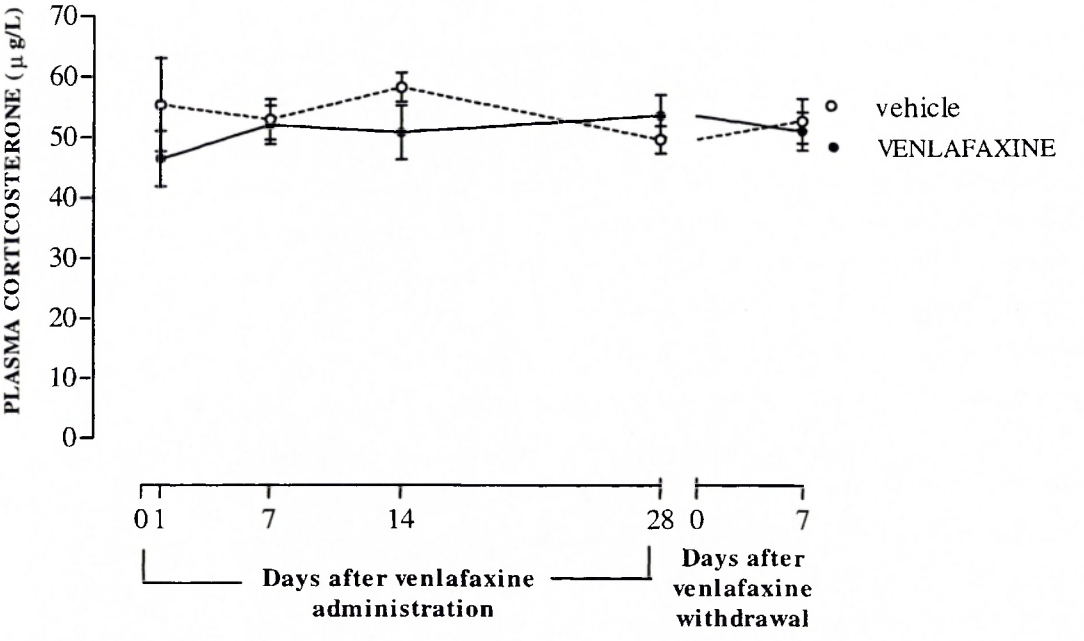
Time course of the effect of venlafaxine administration on B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to CR in rat **hypothalamus.**



Rats (4 per group) received venlafaxine (15 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Hypothalamus tissues were pooled from two animals to increase the reliability of the CR binding assay in such small tissues. Data were expressed as mean ± sem. Mean B_{max} values ranged from 88-142 fmol/mg protein (vehicle groups) and 104–174 fmol/mg protein (venlafaxine groups). Mean K_D values in this region ranged from 1.59-3.44 nM (vehicle groups) and 2.51–3.85 nM (venlafaxine groups). Mean protein values ranged from 102-143 µg/assay (vehicle groups) and 115-144 µg/assay (venlafaxine groups). No statistically significant differences were determined using two-way analysis of variance or Student's t-test where appropriate. See Appendix 3.3(E). ***ANOVA: CR (B_{max})*** *-effect of treatment F (1, 31)=0.04, p=0.84; -effect of time F (3, 31)=1.59, p=0.22; -interaction F(3, 31)=0.99, p=0.42.* ***CR (log K_D)*** *-effect of treatment F (1, 31)=0.16, p=0.69; -effect of time F (3, 31)=0.16, p=0.92; -interaction F (3, 31)=1.11,p=0.37.*

Figure 3.4.4.vi.

Time course of the effect of venlafaxine administration on plasma corticosterone concentrations.



Rats (7-8 per group) received venlafaxine (15 mg/kg, p.o.) or distilled water once daily and were sacrificed after 1, 7, 14 and 28 days of administration and 7 days following withdrawal of the drug (as described in section 3.2). Mean plasma corticosterone concentrations ranged from 49 - 58µg/L (vehicle groups) and 46 - 53 µg/L (paroxetine groups). No statistically significant differences were determined using two-way analysis of variance or Student's t-test. ***ANOVA: log plasma [corticosterone]*** - *effect of treatment F (1, 62) = 2.5, p=0.12;* - *effect of time F (3, 62) = 0.40, p=0.75;* - *interaction F (3, 62) = 1.66, p=0.19.*

3.5. Discussion

The results of the present studies, summarised in Table 3.5, show that the administration of antidepressant drugs in rats produces a complex pattern of alterations in the parameters measured.

Table 3.5. Summary of results

PARAMETER	DMI	PAROXETINE	VENLAFAXINE
BODY WEIGHT	↓ at day 21	---	---
ADRENAL WEIGHT	↓ at days 14 & 21	---	---
CR CORTEX	↑ at day 14	↓ at day 14	---
CR HIPPOCAMPUS	---	---	---
CR STRIATUM	---	↑ at day 7	---
CR HYPOTHALAMUS	---	---	---
CR THYMUS	---	↓ at day 28	---
PLASMA CORTICOSTERONE	↓ 7 days after withdrawal	↑ at day 14 ↓ 7 days after withdrawal	---

↑/↓ indicate significant increases/decreases in B_{max}, --- denotes no change in B_{max} values. All differences displayed were statistically significant at a $p<0.05$ level as compared to vehicle controls.

DMI and corticosteroid receptors

DMI appeared to induce a reduction in body weights when administered for a period of more than 7 days that may be caused by the reported side effect of DMI as an appetite suppressant. The lower adrenal gland weights observed in DMI treated rats after 14 and 21 days of administration are more likely to be due to the reduced body weights and overall smaller size of animals in these groups than DMI-induced alterations.

Although the administration of DMI to adrenally intact rats in the present study did not produce any striking effects on cortical corticosteroid receptor binding parameters, CR numbers were significantly increased at 14 days of DMI administration (Theodorou *et al*, 1997). However, there were no changes at 28 days of administration thus indicating that DMI does not consistently alter ^{3}H-dexamethasone binding to cortical CR.

The observation that CR concentrations in the hippocampus were not affected by DMI administration or withdrawal appear to be in contrast with previous reports that long-term treatment with DMI increases CR binding (+27-42%) and CR mRNA (+42%) concentrations in the hippocampus (Seckl & Fink, 1992; Budziszweska *et al*, 1994a; Okugawa *et al*, 1999).

Acute and long-term administration of tricyclic drugs are found to exert differing effects on monoamine levels in brain tissues with monoaminergic neurotransmission attenuated following chronic exposure (>2 weeks). It is possible that the increase in cortical CR may correspond with a DMI induced reduction in monoamine levels at this time.

As CR levels and biosynthesis are regulated to some degree by NA, it may be that the increase in CR induced by DMI administration is connected with the selective action of DMI on the NA uptake transporter and on monoaminergic transmission in brain tissues. Data pertaining to NA involvement in the modulation of CR is inconsistent. 6-OHDA lesions are demonstrated to reduce CR binding in the hippocampus and PVN but not in the hypothalamus (Weidenfeld *et al*, 1983; 1991) with some studies showing decreased hippocampal MR, but not GR numbers (Yau *et al*, 1992). Lowy (1990) demonstrated the reduction of type II CR in hippocampus, frontal cortex, hypothalamus, lymphocytes

and spleen following reserpine administration and depletion of biogenic amines, possibly via an adrenal independent mechanism as these changes are also observed in adrenalectomised animals.

In contrast, Maccari *et al* (1992) have demonstrated increased hippocampal MRs and hypothalamic GRs following 6-OHDA lesions. Significantly increased hippocampal GR mRNA was observed following chronic DMI administration in DSP4 (*N*-[2-chloroethyl]-*N*-ethyl-2-bromobenzylamine) lesioned rats also supporting noradrenaline-independent regulation of GR mRNA by DMI (Rossby *et al*, 1995). The selective NA uptake inhibitors (desipramine and maprotiline) have also been shown to increase the GR mRNA level in primary cell cultures derived from rat hypothalamus in addition to those derived from the amygdala and/or cerebral cortex which do not contain monoaminergic neurones (Pepin *et al*, 1989). Desipramine has also been found to increase the GR mRNA concentration in mouse fibroblast and neuroblastoma cell cultures. As desipramine is found to increase GR gene promoter activity, it has been suggested that its action may be exerted at the genomic level (Pepin *et al*, 1992a).

Brady *et al* (1991) also demonstrated increased hippocampal MR and decreased levels of GR in the anterior pituitary, along with decreased CRF mRNA in the PVN and tyrosine hydroxylase in the LC following imipramine treatment. It has been suggested that, given the association of major depression with activated CRF and LC-NA systems, the therapeutic efficacy of imipramine may involve time-dependent effects of long-term administration on decreasing hypothalamic CRF mRNA and tyrosine hydroxylase in the LC.

No consistent or significant alterations were observed in plasma corticosterone concentrations following long term DMI administration. Plasma corticosterone were much lower in treated groups 7 days after DMI withdrawal indicating a possible lag effect of DMI suppression of corticosterone output, to some degree, following repeated administration.

Paroxetine and corticosteroid receptors

Cortical CR were significantly decreased following 14 days of paroxetine administration as compared to controls (Maurya *et al*, 1998a). No other studies have reported reductions in CR binding in any brain regions following the administration of paroxetine though some investigations have reported a lack of effect of SSRI's on CR and CR mRNA measures in rat brain (Seckl & Fink, 1992; Budziszweska *et al*, 1994a; 1994b).

B_{max} values of ^{3}H-dexamethasone binding to hippocampal CR were not significantly different from control values following chronic paroxetine administration. These results are in line with those of Budziszweska *et a*l (1994a) who demonstrated that treatment with citalopram (an SSRI) had no effects on CR binding parameters in rat hippocampus. Repeated administration of citalopram did not induce alterations in CR mRNA expression in the rat hippocampus (Seckl & Fink, 1992).

^{3}H-dexamethasone binding to CR in the striatum was elevated after 7 days of paroxetine treatment. Withdrawal of paroxetine appeared to have no effect on CR after 7 days in the cortex, hippocampus or striatum.

In the thymus, CR numbers were significantly reduced after 28 days of paroxetine administration (Maurya *et al*, 1998a). These results suggest that chronic paroxetine administration may induce a reduction of CR numbers in the cortex and also in a peripheral tissue, the thymus. If this is the case, the effects of paroxetine administration in the present study appear to be very region/tissue/time-specific and may be related to differing regulatory mechanisms for CR via different projections and innervations in these areas.

Plasma corticosterone concentrations in paroxetine treated animals were not consistently altered as compared with control groups. However a statistically significant increase in plasma corticosterone was observed at day 14 of paroxetine administration. This corresponds with the time course of the observed reduction in cortical CR and it may be that receptor occupancy by elevated circulating corticosterone concentrations is affecting CR measurements at this time.

Lesions of the 5-HT system using 5, 7-DHT (5, 7-dihydroxytryptamine) have been reported to decrease CR binding in rat hippocampus (Siegel *et al*, 1983). Serotonin and 5-HT_2 agonists have been demonstrated to increase the B_{max} of GR but not MR in hippocampal cell cultures (Mitchell *et al*, 1990; Vedder *et al*, 1993). In contrast to these results, investigations using chloripramine (5-HT reuptake inhibitor) and quipazine (a non-selective 5-HT agonist) have demonstrated reduced CR density in rat hippocampus (Angelucci *et al*, 1982).

The reported effects of 5-HT reuptake blockade depend on the dose and the region studied however, most SSRIs are found to enhance serotonergic neurotransmission (Pineyro & Blier, 1999). The results of the current investigation using paroxetine do not

correspond with previous studies which have reported that elevated 5-HT levels lead to an induction of GR but not MR binding sites in cultured hippocampal cells (Mitchell *et al*, 1990). No up-regulation of CR was observed in any of our experiments however the systems being studied were very different in these investigations therefore the results are difficult to compare.

Venlafaxine and corticosteroid receptors

Specific ^{3}H-dexamethasone binding to CR was not affected by venlafaxine administration in any of the brain regions investigated. Interestingly, the affinity of ^{3}H-dexamethasone binding in the cortex and hippocampus following 14 days of venlafaxine administration was significantly decreased. This appears more likely to be a drug effect rather than a corticosterone effect, as plasma corticosterone was not altered at any point. Withdrawal of venlafaxine for 7 days had no significant effects on CR binding parameters. No changes in CR binding were observed following repeated venlafaxine administration in the thymus. Plasma corticosterone concentrations were unchanged by venlafaxine administration at all investigated time points.

Venlafaxine is reported to display both NA and 5-HT re-uptake inhibition when assessed using an *in vivo* electrophysiological paradigm. However venlafaxine demonstrates a greater potency to inhibit the 5-HT re-uptake process over that of NA in these conditions (Beique *et al*, 1998). Paroxetine and venlafaxine are equipotent to inhibit 5-HT reuptake while DMI is more potent than venlafaxine to block NA reuptake (Beique *et al*, 1998). This property of venlafaxine suggests, in light of our, and other studies with DMI and paroxetine, that its dual NA/5-HT reuptake properties may be exerting opposing actions on CR binding, resulting in no CR alterations following chronic administration.

Most antidepressants affect the same neurotransmitter/receptor systems that are involved in HPA regulation. Modulation of various neurotransmitter systems following antidepressant treatment has also been reported to induce corresponding alterations in some elements of the HPA axis such as 5-HT receptor-mediated ACTH and corticosterone secretion (Li *et al*, 1993). However, though it is clear that the relationship between monoamines (especially 5-HT) and the HPA axis is extremely complex with multiple levels at which the two systems interact with each other, investigations using lesions of the 5-HT and NA systems have yielded conflicting data on changes in corticosteroid nuclear binding in rat hippocampus (Mitchell *et al*, 1990; Budziszweska, 1994b).

The hypothesis that antidepressants may help to maintain GR function in NA and 5-HT neuron-containing cell groups has been proposed by Kitayama *et al* (1988) who found that 2 weeks of imipramine administration increased GR immunoreactivity in the LC and raphe nucleus. This is an interesting proposal as the hippocampus, implicated in CR regulation and antidepressant effects, receives abundant NA and 5-HT innervation again, suggesting an intricate interplay of neurotransmitters and HPA axis components.

Pariante *et al* (1997) showed that desipramine induced GR translocation from the cytoplasm to the nucleus in the absence of steroids and with no effects alone on GR-mediated gene transcription in the mouse fibroblast cell line L929 (displaying NA reuptake-independent effects). Desipramine was also found to potentiate dexamethasone-induced GR translocation and GR-mediated gene transcription in these studies. These authors suggested that an important effect of antidepressants in vivo may involve the facilitation of GR translocation and activation of GR by circulating hormones, possibly via direct effects on one or more heat–shock-proteins (HSPs), thus

facilitating dissociation from the HSP complex. This would have the overall result of increasing GR-mediated feedback inhibition on the HPA axis and reversing corticosteroid hypersecretion in depression. These findings propose a molecular mechanism by which antidepressants normalise HPA abnormalities and imply that GR up-regulation may be a consequence of these rather than their cause.

Several studies have demonstrated the up-regulation of CR binding and CR mRNA following the long-term treatment with tricyclic antidepressants and also reported the lack of effects of SSRI's on CR binding and mRNA expression mostly in the hippocampus (Peiffer *et al*, 1992; Reul *et al*, 1993; Budziszewska, 1994a; 1994b). The present studies have shown no significant changes following treatment with various antidepressants in the hippocampus but have demonstrated some tentative alterations in CR binding parameters in other brain regions and tissues. DMI administration produced an increase in B_{max} values at specific time points – which generally agrees with previous data. B_{max} values at specific times following repeated paroxetine administration were reduced. Venlafaxine had no effects on B_{max} values in any of the brain regions investigated. Since all the antidepressant drugs used in the present study have different mechanisms of action, it could be that their differing effects on CR binding are connected with their actions on different neurotransmitter systems. The increase in CR binding sites following DMI administration implies that NA projections from the locus coeruleus may act as in vivo GR up-regulators. It is also possible that 5-HT potentiation via paroxetine and/or venlafaxine causes very localised or discordant changes in GR expression that are undetectable in whole hippocampus, or other brain regions.

The differing effects of the antidepressants used in these studies on corticosterone output and their correspondence with directions of alterations in CR binding are also of interest. It is possible that during the period of drug administration, changes in both monoaminergic transmission and corticosterone output could be involved in the modulation of brain corticosteroid receptors.

Adrenalectomy and corticosteroid receptors

Though it can be argued that accurate measures of CR can only be obtained in the absence of endogenous corticosteroids (Chou *et al*, 1988; Spencer *et al*, 1990), animals in the present study were not adrenalectomised prior to sacrifice as in all previous studies investigating antidepressant action on brain CR (Reul *et al*, 1993; 1994; Budziszweska *et al*, 1994b). Adrenally intact rats were used in these studies in order to determine the effects of antidepressant action on CR in the presence of endogenous ligand thereby allowing for a closer comparison to clinical observations of CR alterations following stress and antidepressant treatment.

The presence of endogenous ligand in the system at the time of sacrifice would almost certainly affect measures of ^{3}H-dexamethasone binding to CR in the present studies resulting in translocation of more CR (therefore a lower cytosolic availability of CR) than in previous investigations (Spencer *et al*, 1990). In the present studies circulating corticosterone concentrations were kept to minimal levels by sacrificing the animals between 08.00-11.00hrs, at a time when plasma corticosterone levels are lowest (10-15μg/100mL - Shimuzu *et al*, 1983). As mentioned in section 2.12.7, this concentration of circulating plasma corticosterone would result in 70% and 30% type I and II receptor occupancy respectively, thus enabling the majority of CR in tissue preparations from adrenally intact rats to be investigated (Reul & De Kloet, 1985).

The inconsistency of B_{max} and K_D data obtained in the CR binding studies may reflect to some degree, the limitations of the CR radioligand binding assay which has involved the prior adrenalectomy (ADX) of animals to clear endogenous corticosteroids from CR binding sites. However, this procedure would in itself result in the state of the HPA axis being altered before any drugs were administered or measures attempted.

Several components of the HPA axis are changed by ADX. Spinedi *et al* (1991) have demonstrated enhanced ACTH synthesis and secretion following ADX with plasma and anterior pituitary ACTH concentrations increasing with time after ADX. Reul *et al* (1989) have demonstrated a transient increase in both type I and type II CR mRNA following ADX with significantly higher CR levels (2-3 fold higher than control values) at days 1 and 2 after ADX and a return to control values between 8-16 days after ADX. A significant up-regulation of CR (mostly type II receptors) has also been demonstrated in the frontal cortex, hippocampus, hypothalamus lymphocytes and spleen 3 days following ADX of rats (Lowy *et al*, 1990). An up-regulation of GR in the hippocampus and spleen has also been demonstrated several days after ADX (Chao *et al*, 1989; Spencer *et al*, 1991).

Investigations into CR mRNA levels following adrenalectomy have yielded inconsistent data. Reul *et al* (1989) showed a transient increase in hippocampal type I and type II receptor mRNA at 1-3 days post-ADX. No differences in hippocampal CR mRNA regulation were observed in studies by Chao *et al* (1989).

ADX-induced increases in CR binding capacity cannot be attributed to receptor occupation alone as CR levels continue to rise when all measurable plasma corticosterone has cleared and therefore indicates increased numbers of receptor

molecules, a possible consequence of de novo synthesis of receptor protein (Chao *et al*, 1989). The hippocampus, being the primary site of feedback control of corticosteroid hormones would appear to be most susceptible to alterations in CR expression following the removal of endogenous ligand. It appears quite possible therefore that the consistent up-regulation of CR binding and CR mRNA expression demonstrated in various tissues, especially the hippocampus, following repeated administration of antidepressants in ADX animals may be confounded by ADX-induced up-regulation of CR binding sites and CR mRNA expression.

It is apparent that quite different pharmacological effects can act clinically to alleviate depression. Though the notion that up-regulation of brain CR may reveal a common mechanism of action for many antidepressant drugs is gaining credence, in our studies the corresponding alterations of HPA function appear to be related to the properties of the neurotransmitter systems affected by the antidepressant drug. The present study also suggests that various brain regions may respond differently to antidepressant administration and/or corticosterone elevation. Many studies of CR regulation have focused solely on receptor levels in the hippocampus and have suggested that the hippocampus is uniquely sensitive to corticosteroid and stress effects on CR concentrations. The susceptibility of other brain regions or peripheral tissues to corticosteroid receptor down-regulation following chronic antidepressant administration has not been evaluated. Clearly, much more research is required to clarify the complexities of the interactions between antidepressants and components of the HPA axis.

CHAPTER 4

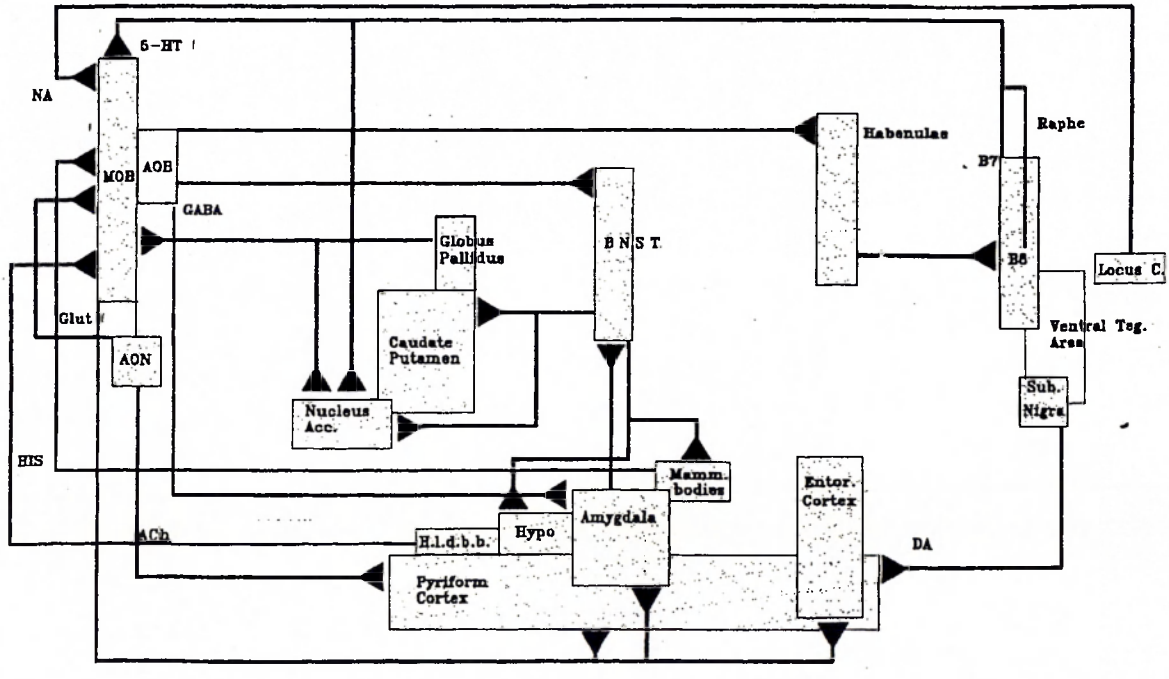
EFFECTS OF ANTIDEPRESSANT ADMINISTRATION ON LOCOMOTOR ACTIVITY AND CORTICOSTEROID RECEPTOR BINDING IN THE OLFACTORY BULBECTOMISED RAT

4.1 Introduction

4.1.1. The olfactory bulbectomised rat model of depression

Bilateral lesioning of the olfactory bulbs produces a variety of changes in the physiology, behaviour, neurochemical, immunological and endocrinological functioning of the rat which are largely reversed by chronic administration of antidepressant drugs (reviewed in Jesberger & Richardson, 1985; Kelly *et al*, 1995). The olfactory system has a highly complex sensory input, which affects a vast number of structures within the brain (see Figure 1). Some degree of denervation would occur in all of these regions following olfactory bulbectomy due to axotomy of cells and trans-neuronal and anterograde degeneration (Brunjes, 1992).

Figure 4.1.1. Schematic representation of the afferent and efferent connections of the olfactory bulbs in the rat.



Ach – acetylcholine; **AON** – anterior olfactory nucleus; **DA** – dopamine; **Entor. Cortex** – entorhinal cortex; **Glut** – glutamate; H.l.d.b.b. – horizontal limb of the diagonal band of Broca; **HIS** – histamine; **Hypo** – hypothalamus; **Locus c.** – locus coeruleus; **Mamm. bodies** – mammilary bodies; **NA** – noradrenaline; **Nucleus acc.** – nucleus accumbens; **Sub. Nigra** – substantia nigra; **Ventral teg. area** – ventral tegmental area. Taken from Kelly *et al*; 1995.

Widespread changes in various parameters induced by olfactory bulbectomy are generally manifested some time after bulb ablation and appear to be irreversible. The effects produced by removal of the olfactory bulbs are due to disruptions in non-sensory central processing rather than anosmia alone as illustrated by studies involving lavage of the nasal cavity with zinc sulphate to produce peripheral anosmia. Rats treated with zinc sulphate in this way were found to be similar to controls in terms of their gross behaviour while bulbectomised rats differed considerably (Alberts & Friedman, 1972; Sieck & Baumbach, 1974).

Rats subjected to olfactory bulbectomy have been reported to display changes in appetite/preference, fluid intake, reduced heart rates and blood pressure and disturbed sleep patterns and thermoregulation (Jancsar & Leonard, 1981; Redmond *et al*, 1995).

The hyperactivity of rats following bulbectomy is a well-documented phenomenon. Many studies have reported that bulbectomised rats displayed increased locomotor and exploratory activity in the open-field arena from two weeks after surgery. Open field hyperactivity was attenuated only by chronic administration of antidepressant drugs (reviewed in Kelly *et al*, 1995; Mundunkotuwa & Horton, 1996). Investigations of home cage activity by O'Halloran *et al* (1993) also revealed hyperactivity of the OB rat during the dark phase of the cycle, which is evident 10 days after surgery. Chronic treatment with imipramine was shown to reverse this hyperactivity in OB rats (Giardinia & Radek, 1991). Several investigations have also shown bulbectomised rats to display deficits in many tasks involving the use of learning and memory including deficits in step-down and step-through passive avoidance tests (Joly & Sanger, 1986, Tiong & Richardson, 1990) and altered performance in one-way active avoidance tasks

(Broekkamp *et al*, 1980) and taste-aversion tests (Jancsar & Leonard, 1981, Redmond *et al*, 1995).

Performance in spatial learning tasks such as the radial maze and Morris water maze is also reportedly impaired in the OB rat providing strong evidence that olfactory bulb ablation affects non-olfactory brain regions (Hall & Macrides, 1983; Kelly *et al*, 1993a; Redmond *et al*, 1994). Recent experiments utilising cocaine-induced place preference also suggested the presence of an anhedonic condition in bulbectomised rats (Calcagnetti *et al*, 1996). Many other behaviours in the rat are impaired by olfactory bulbectomy and reversed by the chronic administration of antidepressants. These include reduced sexual behaviour (Lumia *et al*, 1987), impaired food-motivated behaviours (Kelly & Leonard, 1996) and increased muricidal and cannibalistic behaviour (Iwasaki *et al*, 1985). Many of these behavioural changes are reversed by chronic antidepressant administration however the specificity of some of these behaviours to detect antidepressant activity of drugs is questionable.

4.1.2. Neurochemical alterations in the OB rat

Reports of neurochemical alterations following olfactory bulbectomy have been inconsistent. However, generally NA concentrations and turnover in various brain regions were reduced, (Song & Leonard, 1995), β-adrenoceptor binding was increased (Tiong & Richardson, 1990), central α_2-receptor density was increased (Hong *et al*, 1987) and the growth hormone response to clonidine was blunted (Redmond, 1995).

In addition, 5-HT concentrations were found to be reduced and 5-HIAA concentrations increased in various brain regions of the OB rat (Jancsar & Leonard, 1984; Song & Leonard, 1995). Bulbectomy has also been associated with an increase in the density of

5-HT_{2A} receptors in the frontal cortex (Gurevich *et al*, 1993) and reduced platelet 5-HT uptake and [^{3}H]-imipramine binding sites in the hippocampus (Butler *et al*, 1988). Increased plasma concentrations of α_1-acid glycoprotein (AGP), an allosteric modulator of the 5-HT transporter, have also been reported in both the OB rat and depressed patients (Arnold & Meyerson, 1990; Nemeroff *et al*, 1990). Many altered responses to 5-HT releasing agents and 5-HT receptor agonists have also been reported in the OB rat suggesting changes in mechanisms of 5-HT release and receptor function (McGrath *et al*, 1996 – PhD thesis, NUI). Behavioural normalisation following antidepressant administration has also frequently been correlated with restoration of normal 5-HT function (Mundunkotuwa & Horton, 1996; Grecksch *et a*l, 1997).

In the OB rat, acetycholine concentrations are reduced in the cerebral cortex and increases in choline acetyltransferase activity have been reported, with an increased response to physostigmine demonstrated in some studies suggesting that altered central cholinergic transmission may be involved in the bulbectomy syndrome (Broekkamp *et al*, 1986). Reduced muscarinic receptor binding in several brain regions of the OB rat, including the amygdaloid cortex, hippocampus and hypothalamus (Earley *et al*, 1994) has contrasted with the increased muscarinic receptor binding seen in post-mortem brain tissue from suicide victims (Meyerson *et al*, 1982).

GABA turnover is reportedly increased in the amygdala following olfactory bulbectomy (Jancsar & Leonard, 1984). Dennis *et al* (1993) reported increased $GABA_A$ receptors and decreased $GABA_B$ receptors in the frontal cortex of OB rats. However, some of these deficits are found to normalise with time. The argument for increased GABAergic function in the bulbectomised rat is further supported by reported increases in central benzodiazepine sites (Dennis *et al*, 1995).

Increases in glycine concentrations and decreases in glutamate and aspartate (Schofield *et al*, 1983) have been demonstrated following olfactory bulbectomy implying that an imbalance in these amino acids may form part of the OB syndrome. In recent years, there has been much interest in the involvement of the NMDA (N-methyl-D-aspartate) receptor in the pathology of depression and studies in OB rats also suggest that NMDA-induced responses are increased following bulbectomy (Paul *et al*, 1994; Redmond *et al*, 1995).

Recent studies have claimed that the olfactory bulbectomised rat model of depression shares many immune alterations commonly observed in depressed patients including reduced neutrophil phagocytosis and mitogen-stimulated lymphocyte proliferation (O'Neill & Leonard, 1986; Song *et al*, 1994). Significant reductions in neutrophil catalase activity and increases in superoxide dismutase activity have been demonstrated in bulbectomised rats (Song *et al*, 1994). This study also reported altered differential white blood cell profiles following bulbectomy, consisting of a reduction in the percentage of lymphocytes and an increase in the percentage of neutrophils in the blood.

OB rats are also reported to show increased positive acute phase proteins, reduced negative phase proteins, increased leukocyte aggregation and increased α_1-acid glycoprotein levels, as observed in depressed patients, in addition to reduced weights of the immune-related organs such as the thymus and spleen (Arnold & Meyerson, 1990; Song *et al* 1995).

Considering that olfactory bulbectomy results in a degeneration of neuronal pathways to the amygdala and altered innervation from the amygdala to the hypothalamus,

coupled with alterations in the concentration and turnover of various neurotransmitters involved in the regulation of pituitary hormone release, it is possible that this lesion is associated with changes in HPA-axis function. Lesioning of the olfactory bulbs is reported to produce numerous effects on neuroendocrine parameters however, many studies have yielded inconclusive data with regards to functioning of the HPA-axis in the OB rat.

The HPA-axis of the hypercortisolaemic depressed patient has many features including impaired negative feedback and hypertrophy of the adrenal glands. The most studied endocrine change measured in depression is the hypersecretion of corticosteroids, which is achieved as a result of adaptive changes at several levels of the HPA-axis and the non-suppression of this drive by the administration of dexamethasone (Carroll, 1982; Checkley, 1996). An investigation of endocrine alterations in the OB rat may determine whether the HPA-axis disturbances observed in clinical depression are features that are modelled by the OB rat.

4.1.3. Endocrinological alterations in the olfactory bulbectomised rat

Investigations have shown that bilateral olfactory bulbectomy results in elevation of both basal and stress-induced plasma corticosterone levels that can be reversed by chronic antidepressant administration (Cairncross *et al*, 1977; 1979; Catterelli & Damael, 1986). However, similar studies by Broekkamp (1986) and O'Connor & Leonard (1985) failed to confirm these findings. Other investigations have demonstrated a hypersecretion of corticosterone during the dark phase of the light:dark cycle with normal corticosterone secretion during the light phase (Figure 4.1.1; Song *et al*, 1994) suggesting that there may be an altered periodicity of corticosterone secretion in the OB rat (Marcilhac *et al*, 1997).

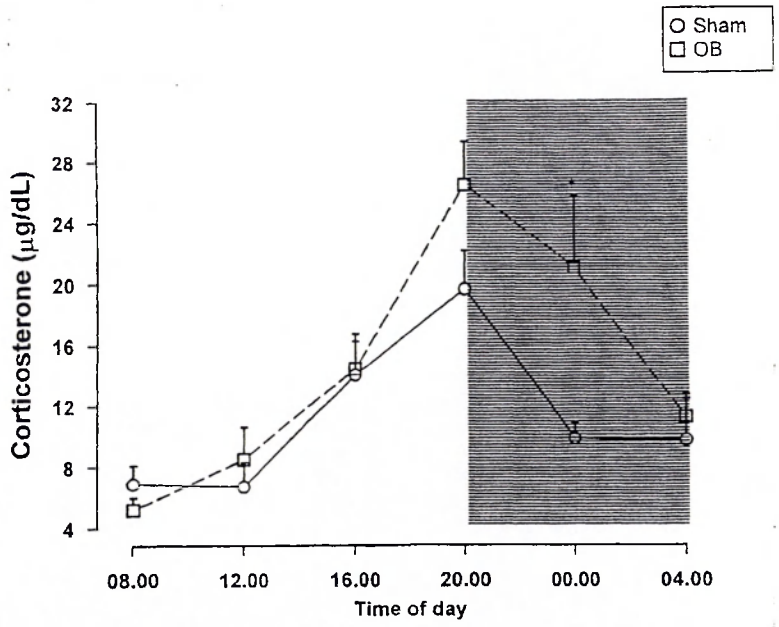


Figure 4.1.2. Circadian pattern of corticosterone secretion following olfactory bulbectomy. 6 weeks after surgery, plasma corticosterone was measured at intervals during the 24-hour cycle. Results are expressed as mean ± sem corticosterone concentration. Shaded area is the dark photoperiod. *$p<0.05$ vs. sham control. n = 5-7. Taken from Kelly *et al*; 1995.

There is considerable evidence that, in humans, altered circadian rhythmicity occurs in association with several categories of depressive disorders. Patterns of rhythm disturbance have also been reported on behavioural, physiological and endocrinological parameters in the OB rat model indicating that bulb lesions may have complex effects on the pituitary-gonadal axis. Bulbectomy was found to affect the circadian rhythm of motor activity in rats (Giardina & Radek, 1991; Marcilhac *et al*, 1999) and mice (Possidente *et al*, 1996) and delayed the onset of entrained activity by over an hour and half (Possidente *et al*, 1990). Circadian rhythms of body temperature were also altered in the OB rat (Marcilhac *et al*, 1997) corresponding to studies in monkeys with maternal separation-induced depression (Reite *et al*, 1982) and the higher body temperatures seen in depressed patients (Pflug *et al*, 1983). Changes in circadian periodicity in OB rats also correspond with reports of increased basal cyclic AMP levels in the suprachiasmatic nucleus (SCN) of the hypothalamus (Vagell *et al*, 1991). This suggests that the observed changes could be due to a direct effect of bulbectomy

on circadian oscillators in the SCN and not as a result of effects on the pituitary-gonadal axis.

Investigations of dexamethasone suppression in the bulbectomised rat found that there were no differences between OB and sham-operated rats (O'Connor, 1985). A modified DST has also been applied to the OB rat (Kelly & Leonard, 1993b) demonstrating suppression of corticosterone output 12 hours after dexamethasone administration under basal and stress-induced conditions. However, 36 hours after dexamethasone administration, the corticosterone surge had returned under basal conditions whereas there appeared to be a prolonged blunting of this effect under stress-induced conditions (see Figure 4.1.2). These results suggest that the OB rat may also differ to controls in its endocrinological response to stress.

Hypertrophy of the adrenal glands has also been demonstrated in bulbectomised rats (Eichelman *et al*, 1972) corresponding to results found in depressed patients (Nemeroff *et al*, 1992).

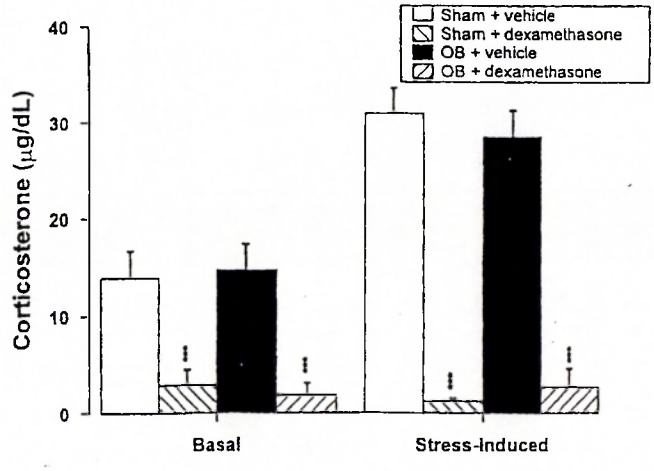


Figure 4.1.3. DST in the OB rat. Two weeks after surgery, rats received dexamethasone (100µg/kg, i.p.). Blood samples were obtained 12 hours later, either under basal or stress-induced conditions. Results are expressed as mean ± sem corticosterone concentration. ***p<0.001 vs. relevant vehicle-treated group. n = 7-12. Taken from Kelly *et al*; 1995.

There is substantial evidence that the olfactory bulbectomised rat fulfils most of the criteria of an animal model of depression. Many of the behavioural, physiological and neurochemical changes observed in the rat following bilateral olfactory bulbectomy appear to reflect symptoms and alterations seen in clinical depression. The bilaterally olfactory bulbectomised rat has been suggested as an appropriate model for depressive illness in that the consequences of disruptions in behavioural and neuronal functioning induced by the lesion may be largely reversed by chronic antidepressant treatment (Jesberger & Richardson, 1985). It appears that the OB rat may also reasonably model some of the neuro-endocrine dysfunction observed in chronically depressed patients with a reversal of some symptoms following chronic antidepressant administration. It would be expected therefore that the OB rat would also exhibit the impaired negative feedback of corticosterone on corticosteroid receptors (via a reduction of MR and GR) commonly observed in depression. However, no studies have been performed in bulbectomised rats as yet to investigate this aspect of HPA-axis function.

The aims of this chapter are to study OB and antidepressant induced effects on both locomotor activity and ^{3}H-dexamethasone binding to hippocampal GR. This will be achieved by;

- the investigation of patterns of locomotor activity (using the open-field arena) following olfactory bulbectomy.
- investigation of the effects of acute and chronic administration of venlafaxine, citalopram, DMI or milnacipran on locomotor activity in the OB rat.
- the investigation of ^{3}H-dexamethasone binding to CR in the hippocampus following olfactory bulbectomy.

- investigation of the effects of acute and chronic administration of venlafaxine, citalopram, DMI or milnacipran. on ^{3}H-dexamethasone binding to hippocampal CR in the OB rat.

4.2 Methods

4.2.1 Animals

Male Sprague-Dawley rats (230-250g; Harlan-Olac, U.K.) were housed 4 per cage (45cm x 25cm x 20cm) under conditions of standard lighting (light period 08.00-20.00hrs) and temperature (20-22°C). A standard laboratory diet was available *ad libitum* except for the periods during which behavioural measurements were taken.

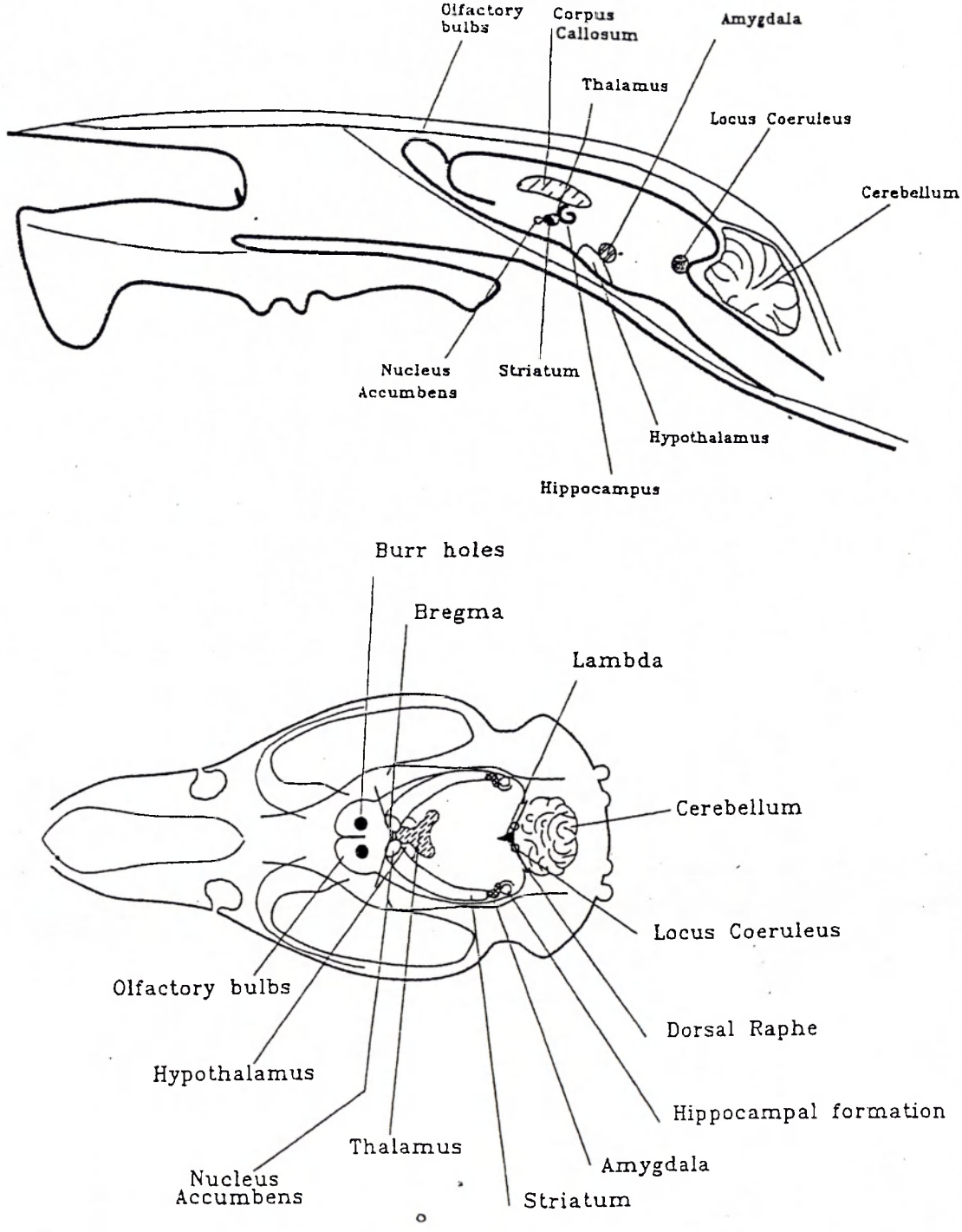
4.2.2 Olfactory bulbectomy

Following a one-week acclimatisation period, bilateral olfactory bulbectomy was performed essentially as described by Cairncross *et al* (1978). Rats were anaesthetised with 2.5% w/v 2-2-2 tribromoethanol (10ml/kg i.p.). The head was shaved and a mid-line sagittal incision was made extending at least 1cm rostral to the bregma, to expose the skull overlying the bulbs. Sufficient pressure was applied to ensure that the periostium on the underlying bone had been penetrated. Two burr holes (2mm in diameter) were drilled through the skull, 5mm rostral to the bregma and 2 mm lateral to the midline, at a point corresponding to the posterior margin of the orbit of the eye (Figure 4.2.2). For sham animals, the dura was carefully pierced and the wound closed. For OB animals the olfactory bulbs, located directly below the burr holes, were removed by aspiration (e.g. by means of a blunt hypodermic needle attached to a water pump). Care was taken to avoid damage to the frontal cortex. Post-operative bleeding was controlled by filling the wound with haemostatic sponge. Oxytetracyline powder was applied to the wound prior to closure with 7.5mm surgical clips. The animals were allowed to recover for 14 days following surgery and were handled daily throughout the recovery period to eliminate any aggressive behaviour that may otherwise arise (Leonard & Tuite, 1981). Following the completion of behavioural studies on the animals, the brains were checked for damage to the frontal cortex and/or incomplete

removal of the olfactory bulbs. Results obtained from any rats displaying the above were excluded from further analysis.

Figure 4.2.2.

A schematic diagram of the location of the burr holes relative to the main anatomical landmarks of the rat skull and the position of the olfactory bulbs in the cranial cavity. Modified from Van Riezen & Leonard, 1991).



4.2.3. Experimental design and drug administration

Two weeks after surgery, the animals were randomly allocated to 6 sets (corresponding to the 6 different time points under investigation) of 10 groups (see Figure 4.2.3):

Group 1: sham + vehicle

Group 2: sham + citalopram (5mg/kg x 2)

Group 3: sham + venlafaxine (15mg/kg x 2)

Group 4: sham + DMI (5mg/kg x 2)

Group 5: sham + milnacipran (15mg/kg x 2)

Group 6: OB + vehicle

Group 7: OB + citalopram (5mg/kg x 2)

Group 8: OB+venlafaxine (10mg/kg x 2)

Group 9: OB + DMI (5mg/kg x 2)

Group10:OB+milnacipran (15mg/kg x 2)

Body weights were recorded regularly throughout the study. Citalopram, venlafaxine, DMI and milnacipran were dissolved in distilled water to give concentrations of 5, 15, 5 and 15 mg/kg respectively. These were administered p.o. in a dosage volume of 1mL/kg, twice a day (08.00-09.00 and 17.00-18.00 hours). Controls received injections of vehicle alone.

Figure 4.2.3.

Experimental design for OB studies

	Day 3	**Day 7**	**Day 10**	**Day 14**	**Day 21**	**Day 28**
Day –22 to –15	Acclim.	Acclim.	Acclim.	Acclim.	Acclim.	Acclim.
Day –15	OB	OB	OB	OB	OB	OB
Day -14 to 0	recovery	recovery	recovery	recovery	recovery	recovery
Day 1 to 3	drug	drug	drug	drug	drug	drug
Day 4	OFT	drug	drug	drug	drug	drug
Day 5-7		drug	drug	drug	drug	drug
Day 8		OFT	drug	drug	drug	drug
Day 9 to 10			drug	drug	drug	drug
Day 11			OFT	drug	drug	drug
Day 12 to 14				drug	drug	drug
Day 15				OFT	drug	drug
Day 16 to 20					drug	drug
Day 21					OFT	drug
Day 22 to 27						drug
Day 28						OFT

- Acclim. = acclimatisation period
- OB = OB surgery
- Drug = drug administration
- OFT = open field testing

All rats were sacrificed immediately following open field testing.

4.3.3. Study 1. b) - ^{3}H-dexamethasone binding to hippocampal CR in OB and SO rats following acute and chronic administration of antidepressant drugs

Due to problems encountered with the Lowry protein assay, B_{max} values are expressed in fmoles/mg tissue as these are more accurate than data expressed as fmoles/mg protein for this particular study.

i) Olfactory bulbectomy

There were no significant differences in the B_{max} or K_D of specific ^{3}H-dexamethasone binding to hippocampal CR from vehicle treated SO and bulbectomised rats (see Tables 4.3.3.B.i-iv).

ii) Citalopram

No significant differences in CR numbers were observed between any of the groups at day 3 of citalopram administration (Table 4.3.3.B.i). At day 28, the citalopram treated group displayed higher B_{max} values than their respective vehicle-treated controls. This difference in specific ^{3}H-dexamethasone binding was significant between OB vehicle and citalopram treated groups and narrowly escaped statistical significance between SO-vehicle and SO-citalopram treated groups. ANOVA revealed a significant interaction between treatment x time for B_{max} values.

No significant differences in K_D values were observed between any of the groups at any time point neither were there any ANOVA interactions between the factors involved for this parameter (Table 4.3.3.B.i).

iii) Venlafaxine

B_{max} values were not significantly altered in any of the groups following 3 days of venlafaxine administration (Table 4.3.3.B.ii). Following 28 days of venlafaxine administration, there was a significant increase in B_{max} values in the SO animals as compared to their vehicle-treated controls. No differences were observed between OB vehicle and venlafaxine treated groups at day 28 of drug administration. ANOVA revealed a no significant interactions of factors for B_{max} values in this study.

There were no differences in K_D values between any of the groups following 3 days of venlafaxine administration however K_D values were significantly increased in the SO-venlafaxine treated group (as compared to SO-vehicle treated group) following 28 days of drug administration. No significant ANOVA interactions were observed between any factors for K_D values.

iv) DMI

There were no significant differences in the B_{max} or K_D of specific ^{3}H-dexamethasone binding to CR in any of the groups at either day 3 or day 28 of DMI administration (Table 4.3.3.B.iii). No significant ANOVA interactions between any of the factors were observed in this study.

v) Milnacipran

B_{max} or K_D values were not significantly altered in any of the groups following either 3 or 28 days of milnacipran administration. A significant ANOVA interaction between drug treatment x lesion was observed for B_{max} values however, no significant ANOVA effects or interactions were observed for K_D values (Table 4.3.2.iv).

Table 4.3.3.B.i

B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to hippocampal CR of sham-operated (SO) and olfactory bulbectomised (OB) rats following acute and chronic administration of **citalopram**.

(A) $\mathbf{B_{max}}$ (fmol/mg tissue)

	SO + vehicle	**SO + citalopram**	**OB + vehicle**	**OB + citalopram**
3 days	2.78 ± 0.3	3.1 ± 0.12	4.1 ± 0.86	2.94 ± 0.26
28 days	2.53 ± 0.28	3.2 ± 0.19	2.88 ± 0.14	3.38 ± 0.14 *

(B) K_D (nM)

	SO + vehicle	**SO + citalopram**	**OB + vehicle**	**OB + citalopram**
3 days	0.95 ± 0.12	1.0 ± 0.12	1.57 ± 0.55	2.3 ± 0.97
28 days	1.32 ± 0.3	1.5 ± 0.21	1.83 ± 0.26	1.8 ± 0.18

Rats (7-10 per group) received citalopram (5mg/kg p.o.) or distilled water twice daily and were sacrificed after 3 and 28 days of administration. CR binding assays were carried out as described in section 2.3. Data are expressed as mean ± sem. Statistically significant differences were determined using ANOVA followed by Students t-tests (GB-STAT v6.0). Statistically significant differences; OB-vehicle vs. OB-citalopram *p<0.05. *ANOVA : CR (B_{max}) – effect of treatment F (1, 53) = 0.11, p=0.74: effect of time F (1, 53) = 0.91, p=0.34; effect of lesion F (1, 53) = 3.08, p=0.085; interaction treatment x time F (1, 53) = 4.3, p=0.04; interaction treatment x lesion F (1, 53) = 2.89, p=0.095; interaction lesion x time F (1, 53) = 0.41, p=0.52; interaction treatment x time x lesion F (1, 53) = 1.85, p=0.18. CR (log K_D) – effect of treatment F (1, 53) = 0.94, p=0.34: effect of time F (1, 53) = 4.26, p=0.04; effect of lesion F (1, 53) = 5.34, p=0.025; interaction treatment x time F (1, 53) = 0.1, p=0.75; interaction treatment x lesion F (1, 53) = 0.01 p=0.92; interaction lesion x time F (1, 53) = 0.008, p=0.93; interaction treatment x time x lesion F (1, 53) = 0.38, p=0.54.*

Table 4.3.3.B.ii

B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to hippocampal CR of sham-operated (SO) and olfactory bulbectomised (OB) rats following acute and chronic administration of **venlafaxine**.

(A) $\mathbf{B_{max}}$ (fmol/mg tissue)

	SO + vehicle	**SO + venlafaxine**	**OB + vehicle**	**OB + venlafaxine**
3 days	2.78 ± 0.3	2.93 ± 0.28	4.1 ± 0.86	3.35 ± 0.39
28 days	2.53 ± 0.28	3.59 ± 0.2**	2.88 ± 0.14	2.97 ± 0.22

(B) K_D (nM)

	SO + vehicle	**SO + venlafaxine**	**OB + vehicle**	**OB + venlafaxine**
3 days	0.95 ± 0.12	1.3 ± 0.3	1.57 ± 0.55	1.8 ± 0.41
28 days	1.32 ± 0.3	2.1 ± 0.23 *	1.83 ± 0.26	1.5 ± 0.24

Rats (7-10 per group) received venlafaxine (15mg/kg p.o.) or distilled water twice daily and were sacrificed after 3 and 28 days of administration. CR binding assays were carried out as described in section 2.3. Data are expressed as mean ± sem. Statistically significant differences were determined using ANOVA followed by Students t-tests (GB-STAT v6.0). Statistically significant differences; SO-vehicle vs. SO-citalopram *p<0.05 and **p<0.01. *ANOVA : CR (B_{max}) – effect of treatment F (1, 54) = 0.28, p=0.6: effect of time F (1, 54) = 1.26, p= 0.27; effect of lesion F (1, 54) = 1.95, p=0.17; interaction treatment x time F (1, 54) = 2.71, p=0.11; interaction treatment x lesion F (1, 54) = 3.12, p=0.08; interaction lesion x time F (1, 54) = 3.62, p=0.06; interaction treatment x time x lesion F (1, 54) = 0.005, p=0.95. CR (log K_D) – effect of treatment F (1, 54) = 1.82, p=0.18: effect of time F (1, 54) = 4.58, p=0.037; effect of lesion F (1, 54) = 0.95, p=0.33; interaction treatment x time F (1, 54) = 0.02, p=0.89; interaction treatment x lesion F (1, 54) = 1.29 p=0.26; interaction lesion x time F (1, 54) = 1.02, p=0.32; interaction treatment x time x lesion F (1, 54) = 2.29, p=0.14.*

Table 4.3.3.B.iii.

B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to hippocampal CR of sham-operated (SO) and olfactory bulbectomised (OB) rats following acute and chronic administration of **DMI.**

(A) $\mathbf{B_{max}}$ (fmol/mg tissue)

	SO + vehicle	**SO + DMI**	**OB + vehicle**	**OB + DMI**
3 days	2.78 ± 0.3	3.65 ± 0.4	4.1 ± 0.86	3.72 ± 0.31
28 days	2.53 ± 0.28	3.13 ± 0.29	2.88 ± 0.14	2.95 ± 0.2

(B) K_D (nM)

	SO + vehicle	**SO + DMI**	**OB + vehicle**	**OB + DMI**
3 days	0.95 ± 0.12	0.9 ± 0.06	1.57 ± 0.55	1.5 ± 0.27
28 days	1.32 ± 0.3	1.7 ± 0.38	1.83 ± 0.26	2.2 ± 0.64

Rats 7-10 per group received DMI (5mg/kg p.o.) or distilled water twice daily and were sacrificed after 3 and 28 days of administration. CR binding assays were carried out as described in section 2.3. Data are expressed as mean ± sem. Statistically significant differences were determined using ANOVA followed by Students t-tests where appropriate (GB-STAT v6.0) however, no statistically significant differences were observed in this study. *ANOVA : CR (B_{max}) – effect of treatment F (1, 54) = 1.12, p=0.29: effect of time F (1, 54) = 6.34, p= 0.015; effect of lesion F (1, 54) = 2.04, p=0.16; interaction treatment x time F (1, 54) = 0.03, p=0.87; interaction treatment x lesion F (1, 54) = 2.68, p=0.11; interaction lesion x time F (1, 54) = 1.26, p=0.27; interaction treatment x time x lesion F (1, 54) = 0.43, p=0.51. CR (log K_D) – effect of treatment F (1, 54) = 0.49, p=0.49: effect of time F (1, 54) = 5.85, p=0.019; effect of lesion F (1, 54) = 4.16, p=0.046; interaction treatment x time F (1, 54) = 0.005, p=0.94; interaction treatment x lesion F (1, 54) = 0.018 p=0.89; interaction lesion x time F (1, 54) = 0.079, p=0.78; interaction treatment x time x lesion F (1, 54) = 0.64, p=0.43.*

Table 4.3.3.B.iv.

B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to hippocampal CR of sham-operated (SO) and olfactory bulbectomised (OB) rats following acute and chronic administration of **milnacipran.**

(A) $\mathbf{B_{max}}$ (fmol/mg tissue)

	SO + vehicle	**SO + milnacipran**	**OB + vehicle**	**OB + milnacipran**
3 days	2.78 ± 0.3	2.94 ± 0.25	4.1 ± 0.86	2.88 ± 0.15
28 days	2.53 ± 0.28	3.1 ± 0.31	2.88 ± 0.14	2.54 ± 0.31

(B) K_D (nM)

	SO + vehicle	**SO + milnacipran**	**OB + vehicle**	**OB + milnacipran**
3 days	0.95 ± 0.12	1.1 ± 0.12	1.57 ± 0.55	1.1 ± 0.15
28 days	1.32 ± 0.3	2.1 ± 0.36	1.83 ± 0.26	1.6 ± 0.28

Rats 7-10 per group received milnacipran (15mg/kg p.o.) or distilled water twice daily and were sacrificed after 3 and 28 days of administration. CR binding assays were carried out as described in section 2.3. Data are expressed as mean ± sem. Statistically significant differences were determined using ANOVA followed by Students t-tests where appropriate (GB-STAT v6.0) however, no statistically significant differences were observed in this study. ***ANOVA : CR (B_{max})*** *– effect of treatment F (1, 53) = 0.62, p=0.44: effect of time F (1, 53) = 2.42, p= 0.13; effect of lesion F (1, 53) = 1.0, p=0.32; interaction treatment x time F (1, 53) = 1.49, p= 0.23; interaction treatment x lesion F (1, 53) = 4.7, p=0.035; interaction lesion x time F (1, 53) = 1.97, p=0.17; interaction treatment x time x lesion F (1, 53) = 0.19, p=0.66.* ***CR (log K_D)*** *– effect of treatment F (1, 53) = 0.74, p=0.39: effect of time F (1, 53) = 10.8, p=0.0018; effect of lesion F (1, 53) = 0.65, p=0.42; interaction treatment x time F (1, 53) = 0.36. p= 0.55; interaction treatment x lesion F (1, 53) = 2.86 p=0.097; interaction lesion x time F (1, 53) = 0.15, p=0.7; interaction treatment x time x lesion F (1, 53) = 0.96, p=0.33.*

4.3.4. Study 2. a) - Determination of open field activity in SO rats following 3, 7, 10, 14, 21 and 28 days of antidepressant administration.

i) Citalopram

In sham-operated rats, citalopram administration over a period of 3 – 28 days did not significantly alter locomotor activity as compared to respective vehicle treated controls (Table 4.3.4.i.A). Ambulation scores were significantly reduced over time in both vehicle and citalopram administered groups, as were rearing scores. A significant elevation of rearing scores was observed in SO-citalopram treated animals as compared to the SO-vehicle treated group following 14 days of drug administration (Table 4.3.4.i.B). Grooming and defecation scores were not consistent and are not displayed (see Appendix 4.3.4.i). No significant interactions of time x drug treatment were observed in ambulation or rearing scores following ANOVA in this study however, each factor contributed a significant effect separately.

ii) Venlafaxine

There were no differences in ambulation scores between vehicle and venlafaxine treated groups at any of the time points although ambulation scores were reduced over time in both groups (Table 4.3.4.ii.A). No significant changes in rearing scores were observed in any of the groups following venlafaxine administration (Table 4.3.4.ii.B). No significant ANOVA interactions between time x drug treatment were observed in this study for ambulation or rearing however, a significant effect of time was observed on both of these parameters.

iii) DMI

Locomotor activity as measured by ambulation scores was not significantly altered by DMI administration over 28 days as compared to vehicle treated controls (Table 4.3.4.iii.A). There were also no significant differences observed in rearing scores following DMI treatment or over time (Table 4.3.4.iii.B). No significant ANOVA interactions were observed in this study for either ambulation or rearing scores.

iv) Milnacipran

There were no differences in locomotor activity between vehicle and milnacipran treated groups at any of the time points. Changes observed in both ambulation and rearing scores were very inconsistent in the milnacipran treated group over time (Table 4.3.4.iv.A). Rearing scores were also significantly reduced over time in the milnacipran treated group (Table 4.3.4.iv.B). Elevated rearing scores were observed in the milnacipran treated group at day 14 of drug administration as compared to vehicle-treated controls. No significant ANOVA interactions between factors were observed in this analysis for either ambulation or rearing scores.

Table 4.3.4.i.

Time course of the effects of vehicle or **citalopram** administration on locomotor activity (A) and rearing (B) in sham-operated (SO) rats.

(A) Ambulation

	SO + vehicle	SO + citalopram
Day 3	78 ± 5	82 ± 12
Day 7	78 ± 7	89 ± 10
Day 10	82 ± 7	96 ± 5
Day 14	74 ± 10	86 ± 6
Day 21	50 ± 10	67 ± 4
Day 28	48 ± 6	53 ± 12

(B) Rearing

	SO + vehicle	SO + citalopram
Day 3	18 ± 3	14 ± 3
Day 7	19 ± 3	24 ± 4
Day 10	19 ± 2	25 ± 2
Day 14	16 ± 3	24 ± 2 *
Day 21	9 ± 3	16 ± 3
Day 28	9 ± 3	13 ± 3

Rats (7-10 per group) received citalopram (5mg/kg p.o.) or distilled water twice daily and were tested for open-field activity approximately 16 hours following the last dose as described in section 4.2.4. Data are expressed as mean ± sem. Statistically significant differences were determined using ANOVA followed by Students t-test where appropriate and are denoted by * $p<0.05$. ***One-way ANOVA - Ambulation** in SO-vehicle $F(5, 49) = 3.9$, $p=0.005$; SO-citalopram $F(5, 50) = 3.22$, $p=0.015$. **Rearing** in SO-vehicle $F(5, 49) = 2.72$, $p=0.03$; SO-citalopram $F(5, 50) = 3.81$, $p=0.006$. **ANOVA : Ambulation** – effect of treatment $F(1, 100) = 5.04$, $p=0.027$: effect of time $F(5, 100) = 6.96$, $p<0.0001$; interaction treatment x time $F(5, 100) = 0.21$, $p= 0.96$. **Rearing** – effect of treatment $F(1, 98) = 5.84$, $p=0.18$: effect of time $F(5, 98) = 5.08$, $p=0.0004$; interaction treatment x time $F(5, 100) = 1.08$. $p= 0.38$.*

Table 4.3.4.ii.

Time course of the effects of vehicle or **venlafaxine** administration on locomotor activity (A) and rearing (B) in sham-operated (SO) rats.

(A) Ambulation

	SO + vehicle	SO + venlafaxine
Day 3	78 ± 5	86 ± 14
Day 7	78 ± 7	87 ± 14
Day 10	82 ± 7	97 ± 11
Day 14	74 ± 10	76 ± 10
Day 21	50 ± 10	50 ± 12
Day 28	48 ± 6	48 ± 12

(B) Rearing

	SO + vehicle	SO + venlafaxine
Day 3	18 ± 3	19 ± 4
Day 7	19 ± 3	21 ± 3
Day 10	19 ± 2	25 ± 2
Day 14	16 ± 3	22 ± 4
Day 21	9 ± 3	12 ± 3
Day 28	9 ± 3	13 ± 3

Rats (7-10 per group) received venlafaxine (15mg/kg p.o.) or distilled water twice daily and were tested for open-field activity approximately 16 hours following the last dose as described in section 4.2.4. Data are expressed as mean ± sem. Statistically significant were determined using ANOVA followed by Students t-tests where appropriate. ***One-way ANOVA - Ambulation*** *in SO-vehicle F (5, 49) =3.9, p=0.005; SO-venlafaxine F (5, 47) =2.79, p=0.029.* ***Rearing*** *in SO-vehicle F (5, 49) =2.72, p=0.03; SO-venlafaxine F (5, 47) = 2.54, p=0.043.* ***ANOVA : Ambulation*** *– effect of treatment F (1, 97) = 1.0, p=0.32: effect of time F (5, 97) = 6.23, p<0.0001; interaction treatment x time F (5, 97) = 0.18, p= 0.97.* ***Rearing*** *– effect of treatment F (1, 94) = 4.56, p=0.036: effect of time F (5, 94) = 5.2, p=0.0003; interaction treatment x time F (5, 94) = 0.34. p= 0.89.*

Table 4.3.4.iii.

Time course of the effects of vehicle or **DMI** administration on locomotor activity (A) and rearing (B) in sham-operated (SO) rats.

(A) Ambulation

	SO + vehicle	SO + DMI
Day 3	78 ± 5	81 ± 10
Day 7	78 ± 7	69 ± 6
Day 10	82 ± 7	73 ± 5
Day 14	74 ± 10	71 ± 6
Day 21	50 ± 10	47 ± 10
Day 28	48 ± 6	58 ± 12

(B) Rearing

	SO + vehicle	SO + DMI
Day 3	18 ± 3	17 ± 3
Day 7	19 ± 3	21 ± 2
Day 10	19 ± 2	20 ± 3
Day 14	16 ± 3	20 ± 3
Day 21	9 ± 3	12 ± 3
Day 28	9 ± 3	10 ± 4

Rats (7-10 per group) received DMI (5mg/kg p.o.) or distilled water twice daily and were tested for open-field activity approximately 16 hours following the last dose as described in section 4.2.4. Data are expressed as mean ± sem. Statistically significant differences were determined using ANOVA followed by Students t-tests where appropriate. ***One-way ANOVA - Ambulation*** *in SO-vehicle F (5, 49) =3.9, p=0.005; SO-DMI F (5, 51) =2.02, p=0.093.* ***Rearing*** *in SO-vehicle F (5, 49) =2.72, p=0.03; SO-DMI F (5, 51) = 2.27, p=0.063.* ***ANOVA : Ambulation*** *– effect of treatment F (1, 101) = 0.13, p=0.72: effect of time F (5, 101) = 5.7, p=0.0001; interaction treatment x time F (5, 101) = 0.44, p= 0.82.* ***Rearing*** *– effect of treatment F (1, 98) = 0.73, p=0.4: effect of time F (5, 98) = 4.32, p=0.0015; interaction treatment x time F (5, 98) = 0.23, p= 0.95.*

Table 4.3.4.iv.

Time course of the effects of vehicle or **milnacipran** administration on locomotor activity (A) and rearing (B) in sham-operated (SO) rats.

(A) Ambulation

	SO + vehicle	SO + milnacipran
Day 3	78 ± 5	75 ± 11
Day 7	78 ± 7	75 ± 9
Day 10	82 ± 7	96 ± 11
Day 14	74 ± 10	97 ± 9
Day 21	50 ± 10	56 ± 10
Day 28	48 ± 6	63 ± 11

(B) Rearing

	SO + vehicle	SO + milnacipran
Day 3	18 ± 3	14 ± 3
Day 7	19 ± 3	19 ± 3
Day 10	19 ± 2	23 ± 2.3
Day 14	16 ± 3	27 ± 4 *
Day 21	9 ± 3	9 ± 2
Day 28	9 ± 3	15 ± 2

Rats (7-10 per group) received milnacipran (15mg/kg p.o.) or distilled water twice daily and were tested for open-field activity approximately 16 hours following the last dose as described in section 4.2.4. Data are expressed as mean ± sem. Statistically significant differences were determined using ANOVA followed by Student's t-tests where appropriate and are denoted by * $p<0.05$. ***One-way ANOVA - Ambulation*** *in SO-vehicle $F(5, 49) = 3.9$, $p=0.005$; SO-milnacipran $F(5, 47) = 2.7$, $p=0.033$.* ***Rearing*** *in SO-vehicle $F(5, 49) = 2.72$, $p=0.03$; SO-milnacipran $F(5, 47) = 5.55$, $p=0.0005$.* ***ANOVA : Ambulation*** *– effect of treatment $F(1, 97) = 2.8$, $p=0.098$: effect of time $F(5, 97) = 5.79$, $p=0.0001$; interaction treatment x time $F(5, 97) = 0.67$, $p= 0.65$.* ***Rearing*** *– effect of treatment $F(1, 94) = 2.34$, $p=0.13$: effect of time $F(5, 94) = 5.47$, $p=0.0002$; interaction treatment x time $F(5, 94) = 1.79$, $p= 0.12$.*

4.3.4. Study 2. b) - ^{3}H-dexamethasone binding to hippocampal CR in SO rats following antidepressant administration for 3, 7, 10, 14, 21 and 28 days.

i) Citalopram

Specific ^{3}H-dexamethasone binding to hippocampal CR was not significantly altered following citalopram administration over 28 days in SO animals (Figure 4.3.4.i.A). ANOVA revealed significant separate effects of treatment and time however there was no interaction between factors. The K_D of specific ^{3}H-dexamethasone binding to CR was also unchanged by citalopram administration over 28 days in SO animals (Figure 4.3.4.i.B). There were no significant ANOVA interactions between factors for K_D values. All CR binding values are displayed in Appendix 4.3.4.ii.

ii) Venlafaxine

No differences were observed in the B_{max} of ^{3}H-dexamethasone binding to CR over 21 days of venlafaxine administration. A significant increase (+30%) in B_{max} was observed following 28 days of venlafaxine administration as compared to vehicle treated controls (Figure 4.3.4.ii.A). ANOVA revealed significant effects of time and treatment separately but no interaction between factors. K_D values were not significantly altered by venlafaxine administration for up to 21 days but were significantly increased in the venlafaxine treated group at day 28 of drug administration (Figure 4.3.4.ii.B.). There were no significant ANOVA effects or interactions of factors on K_D values in this study.

iii) DMI

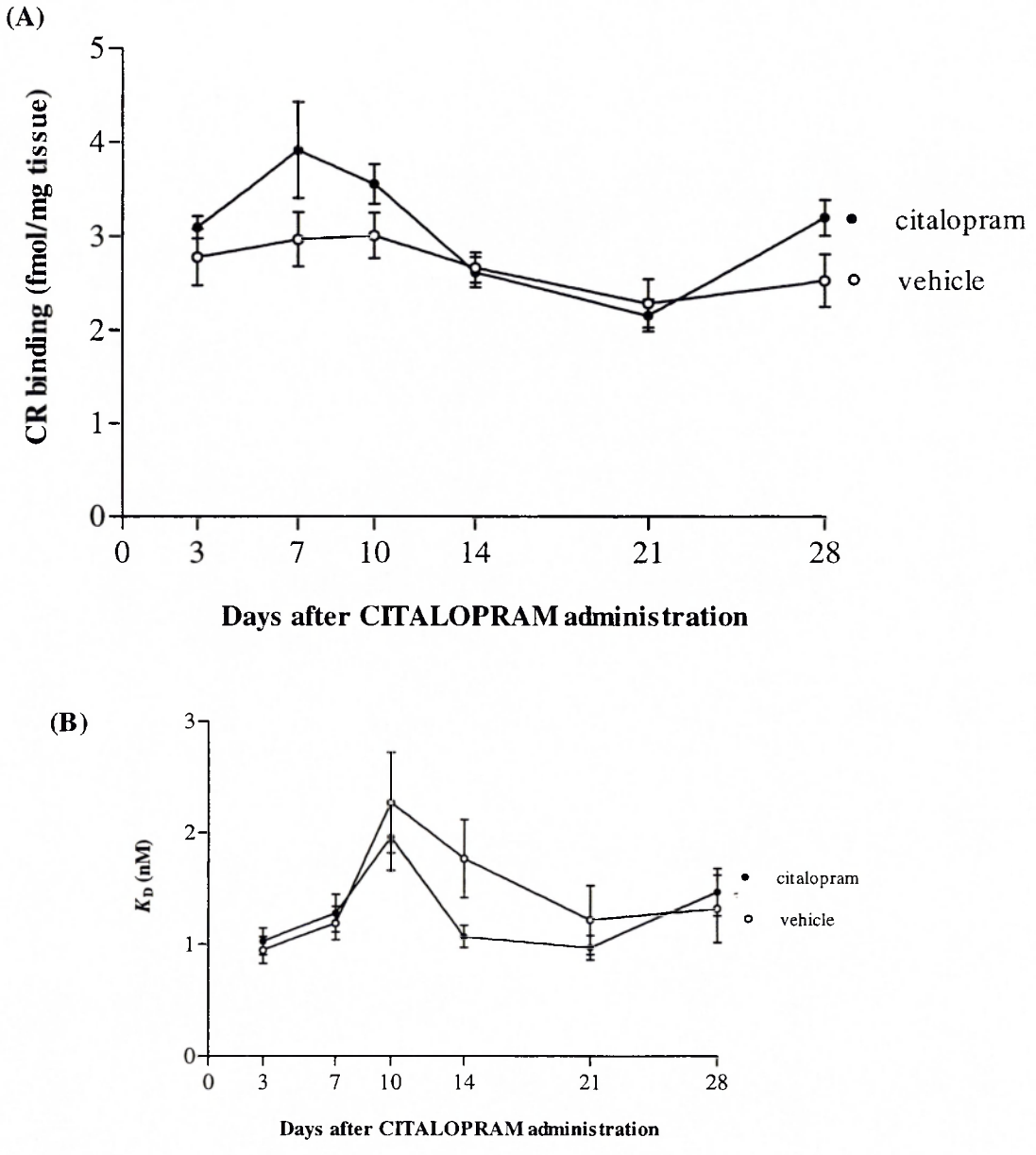
DMI administration for up to 28 days did not significantly alter specific ^{3}H-dexamethasone binding to CR in hippocampal tissue from SO rats (Figure 4.3.4.iii.A). ANOVA revealed significant effects of time and treatment separately but no interaction between factors. Overall, K_D values were not significantly altered in DMI treated rats (Figure 4.3.4.iii.B). There were no significant ANOVA effects or interactions of factors on K_D values in this study.

iv) Milnacipran

Administration of milnacipran had no significant effects on specific ^{3}H-dexamethasone binding to hippocampal CR (Figure 4.3.4.iv.A). K_D values were not affected by milnacipran treatment (see Figure 4.3.4.iv.B). No interaction of factors was observed on either parameter in this study.

Figure 4.3.4.i.

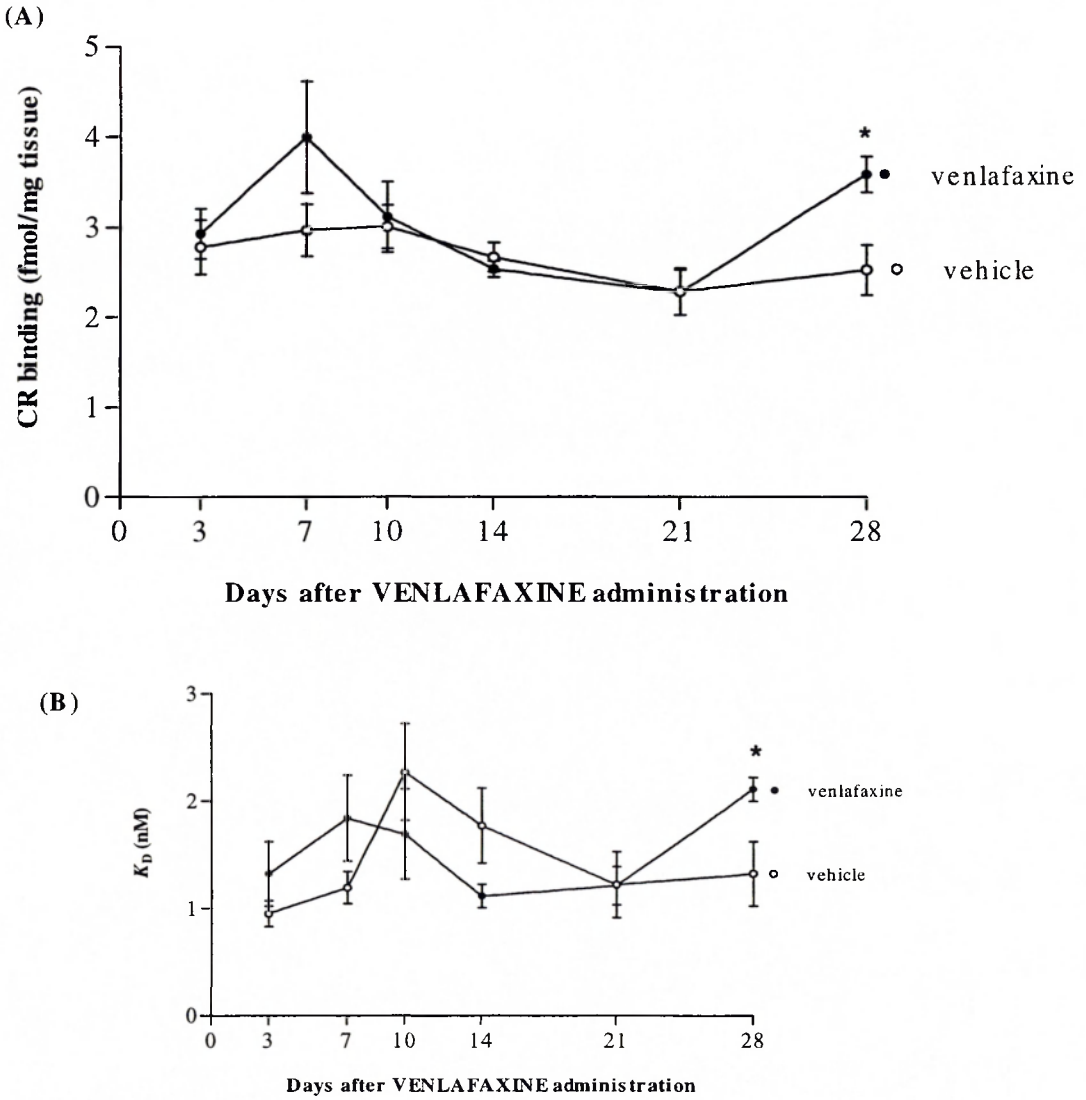
Time course of the effect of **citalopram** administration on the B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to hippocampal CR in sham-operated (SO) rats.



SO rats (6-8 per group) received citalopram (5mg/kg p.o.) or distilled water twice daily and were sacrificed after 3, 7, 10, 14, 21 and 28 days of administration. Data are expressed as mean ±sem. Mean B_{max} values ranged from 2.3-3.01 fmol/mg tissue (vehicle groups) and 2.16-3.92 fmol/mg tissue (citalopram groups). Mean K_D values ranged from 2.1-2.9 nM (vehicle groups) and 2.1-3.8 (citalopram groups). Statistically significant differences were determined using ANOVA followed by Students t-tests where appropriate and are denoted by *p<0.05. See Appendix 4.3.4.ii. ***ANOVA: CR (B_{max})*** *- effect of treatment F (1, 94) = 6.48, p=0.013; effect of time F (5, 94) = 5.7, p=0.0001; interaction treatment x time F (5, 94) = 1.3, p= 0.27.* ***CR (logK_D)*** *- effect of treatment F (1, 94) = 0.38, p=0.54; effect of time F (5, 94) = 5.22, p=0.0003; interaction treatment x time F (5, 94) = 0.87, p=0.51.*

Figure 4.3.4.ii.

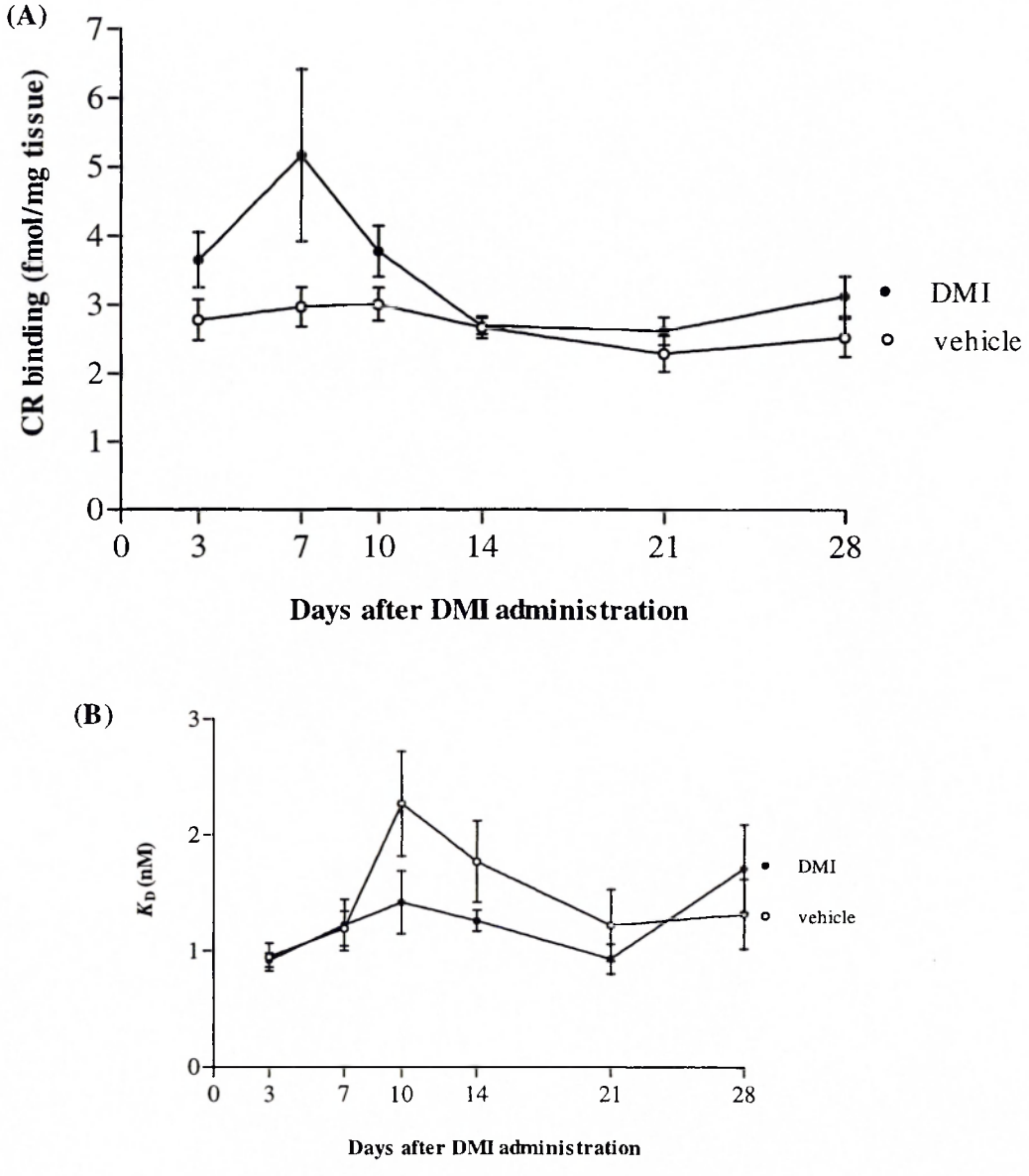
Time course of the effect of **venlafaxine** administration on the B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to hippocampal CR in sham-operated (SO) rats.



SO rats (6-8 per group) received venlafaxine (15mg/kg p.o.) or distilled water twice daily and were sacrificed after 3, 7, 10, 14, 21 and 28 days of administration. Data are expressed as mean ±sem. Mean B_{max} values ranged from 2.3-3.01 fmol/mg tissue (vehicle groups) and 2.28-4.0 fmol/mg tissue (venlafaxine groups). Mean K_D values ranged from 2.1-2.9 nM (vehicle groups) and 2.24-3.68 (venlafaxine groups). Statistically significant differences were determined using ANOVA followed by Students t-tests where appropriate and are denoted by *p<0.05. See Appendix 4.3.4.ii. ***ANOVA: CR*** ***(B_{max})*** *- effect of treatment F (1, 94) = 4.29, p=0.042; effect of time F (5, 94) = 3.66, p=0.0048; interaction treatment x time F (5, 94) = 1.5, p= 0.2.* ***CR (logK_D)*** *- effect of treatment F (1, 94) = 0.3, p=0.58; effect of time F (5, 94) = 2.28, p=0.054; interaction treatment x time F (5, 94) = 2.0, p=0.087.*

Figure 4.3.4.iii.

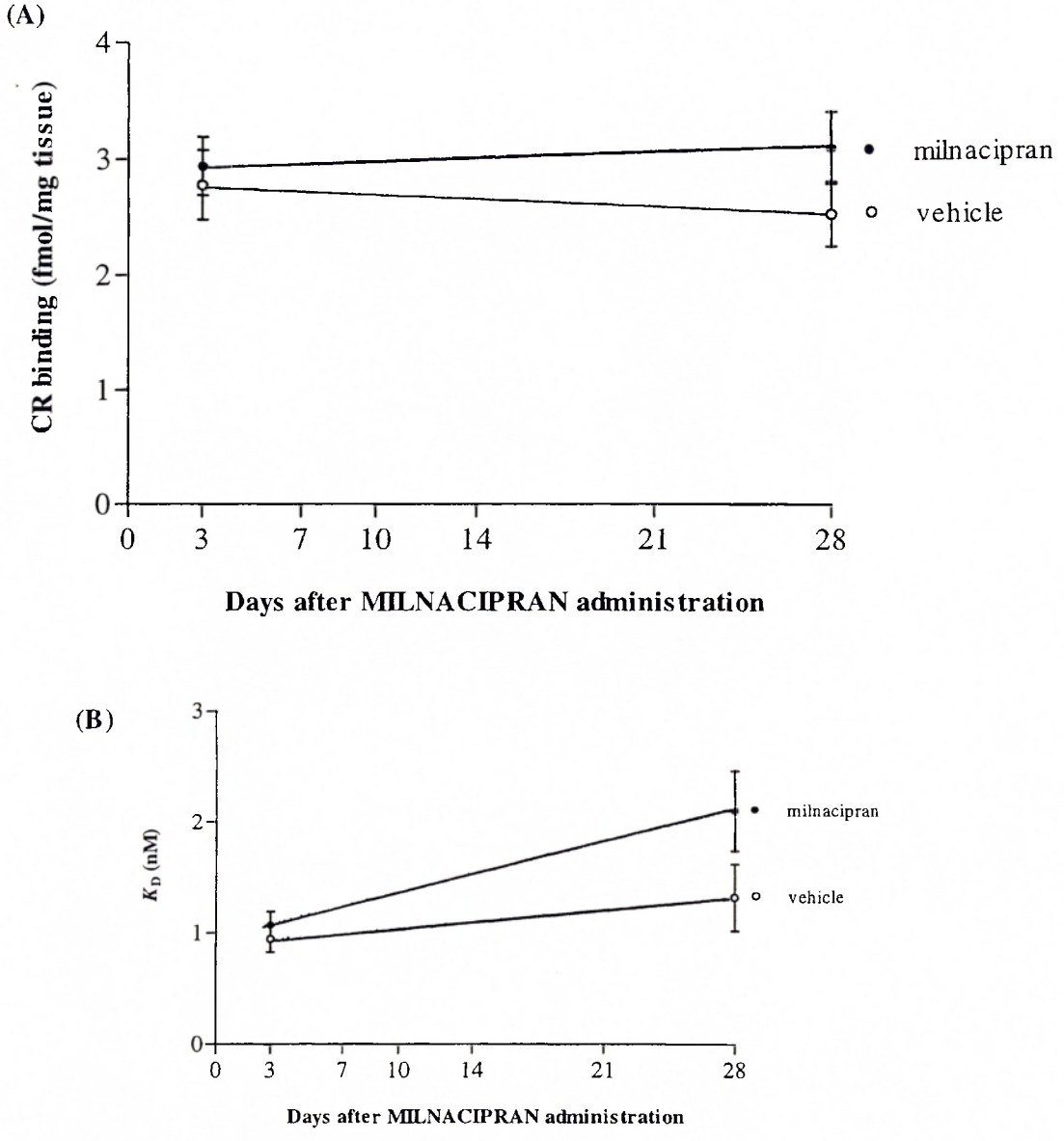
Time course of the effect of **DMI** administration on the B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to hippocampal CR in sham-operated (SO) rats.



SO rats (6-8 per group) received DMI (5mg/kg p.o.) or distilled water twice daily and were sacrificed after 3, 7, 10, 14, 21 and 28 days of administration. Data are expressed as mean ±sem. Mean B_{max} values ranged from 2.3-3.01 fmol/mg tissue (vehicle groups) and 2.62-5.17 fmol/mg tissue (DMI groups). Mean K_D values ranged from 2.1-2.9 nM (vehicle groups) and 2.52-4.94 (DMI groups). Statistically significant differences were determined using ANOVA followed by Students t-tests where appropriate and are denoted by *p<0.05. See Appendix 4.3.4.ii. ***ANOVA: CR (B_{max})*** *- effect of treatment F (1, 92) = 12.88, p=0.0006; effect of time F (5, 92) = 4.59, p=0.001; interaction treatment x time F (5, 92) = 1.92, p= 0.10.* ***CR ($logK_D$)*** *- effect of treatment F (1, 92) = 1.41, p=0.24; effect of time F (5, 92) = 3.43, p=0.0074; interaction treatment x time F (5, 92) = 1.06, p=0.39.*

Figure 4.3.4.iv.

Time course of the effect of **milnacipran** administration on the B_{max} (A) and K_D (B) of specific ^{3}H-dexamethasone binding to hippocampal CR in sham-operated (SO) rats.



SO rats (6-8 per group) received milnacipran (15mg/kg p.o.) or distilled water twice daily and were sacrificed after 3, 7, 10, 14, 21 and 28 days of administration. Data are expressed as mean ±sem. Mean B_{max} values ranged from 2.53-2.78 fmol/mg tissue (vehicle groups) and 2.94-3.1 fmol/mg tissue (DMI groups. Mean K_D values ranged from 2.36-2.84 nM (vehicle groups) and 2.82-3.01 (DMI groups). Statistically significant differences were determined using ANOVA followed by Students t-tests where appropriate and are denoted by *p<0.05. See Appendix 4.3.4.ii. ***ANOVA: CR (B_{max})*** *- effect of treatment F (1, 30) = 1.6, p=0.22; effect of time F (1, 30) = 0.02, p=0.89; interaction treatment x time F (1, 30) = 0.52, p= 0.48.* ***CR ($logK_D$)*** *- effect of treatment F (1, 30) = 4.59, p=0.04; effect of time F (1, 30) = 9.51, p=0.0047; interaction treatment x time F (1, 30) = 1.75, p=0.2*

4.4. Discussion

4.4.1 Effects of olfactory bulbectomy and antidepressant administration on body weights.

Olfactory bulbectomy did not significantly affect the body weights of rats during this investigation. A previous study (McGarvey, 1996) has also shown that there are no differences in body weights between un-operated (naïve), SO and OB rats.

Administration of citalopram, venlafaxine and milnacipran over 28 days also had no significant effects on body weights. However, long-term DMI administration induced a decrease in weight gain in the SO group. This observation is in agreement with other findings (see section 3.4.) and may reflect the side effect of DMI as an appetite suppressant (Frank *et al*, 1990; Redmond *et al*, 1995).

4.4.2. Effects of olfactory bulbectomy and antidepressant administration on locomotor activity.

Study 1 confirmed the earlier findings that removal of the olfactory bulbs in rats leads to increased activity in the open field arena (Sieck *et al*, 1974; Van Riezen & Leonard, 1990). Although it was originally believed that these behavioural differences were due to loss of olfaction, it is now apparent that these changes are a consequence of altered function of brain areas innervated by the olfactory bulbs, particularly the limbic system, since animals rendered peripherally anosmic with zinc sulfate do not display these behavioural changes (Alberts & Friedman, 1972; Sieck & Baumbach, 1974).

Defecation scores in the open field were found to be an unreliable parameter for OB deficits therefore were not commented upon. Rearing scores, taken as supplementary measures, were not generally significantly altered by bulbectomy or antidepressant

administration. Rearing appeared to follow a similar pattern to ambulation with some elevated scores observed in OB-vehicle treated groups, these sometimes being significantly attenuated in antidepressant administered OB groups. However, these effects were generally found to be statistically insignificant.

Many investigators have reported on the increase in locomotor activity of OB rats in novel environments and increased exploration in the open-field arena. It has been suggested that activity and emotionality are highly correlated in the open field (Richman *et al*, 1972) and the following factors may be involved in this aspect of dysfunctional behaviour in the OB rat.

Many rodents respond quite specifically to odours emitted or deposited by previous animals. Olfactory bulbectomy reduces information received thereby decreasing the rats knowledge of the environment and making it more unpredictable. In the OB rat, this information is absent and consequently, less time is spent investing odour sources and more time is spent in active movement. It has been suggested that due to its distorted perception, the OB rat is more apprehensive of the novel environment and reacts to each new stimulus as if it is threatening (e.g.; rat escapes from A to B and upon finding B no more secure than A, moves to C, etc...) thus resulting in increased activity (Leonard & Tuite, 1981).

Removal of important sources of information by bilateral olfactory bulbectomy drastically reduces information-processing efficiency. The OB rat therefore, lacking a complete sensory input, displays impaired habituation to a novel environment and an inability to adequately assess the rate of environmental change. Control rats placed in the open-field display a progressive reduction in activity over successive tests (this was

observed even in the SO groups in the preceding studies) however, OB rats display and maintain high activity levels even after twenty exposures (Kenshalo & Isaac, 1977).

The deficits demonstrated in passive avoidance tasks in OB rats also support this hypothesis as, when placed in the novel environment of the passive avoidance paradigm, the immediate response of the OB rat is to escape, resulting in deficient acquisition of this task as compared to SO controls (Leonard & Tuite, 1981). The superiority of OB rats over controls in acquisition of active avoidance tasks also lends support to this hypothesis (Sieck *et al*, 1972; 1974) though this observation is not a consistent one (King & Cairncross, 1974).

Since the open field test only provides a very short-term observation of locomotor activity, some investigators have focussed on home cage activity as an index of activity level. Using continuous monitoring in the home cage activity box, several studies have demonstrated increased activity of OB animals but only in the dark phase of the activity cycle (Giardina & Radek, 1991; O'Halloran *et al,* 1993; Marcilhac *et al*; 1997).

In addition to altered 'emotional' behaviour, deficits in cognitive functioning of the OB rat such as those tested using the radial arm maze and Morris water maze (Hall & Macrides, 1983; Redmond *et al*, 1994) have also been reported. These tests are specifically designed to investigate the function of the septohippocampal system and the observed deficits are attenuated by chronic antidepressant administration (Redmond *et al*, 1994) providing strong evidence that olfactory bulbectomy disrupts non-olfactory brain regions.

The relationship between activity and emotionality in the open field in these studies supports the hypothesis of a greater stress response in animals following olfactory bulbectomy than in naïve or SO animals.

In our investigations, OB-induced hyperactivity was significantly attenuated only following chronic treatment with citalopram, venlafaxine, DMI and milnacipran. Acute administration of these antidepressants had no effect on this parameter (Maurya *et al*, 1998b). These findings are consistent with the results of previous studies of the effects of repeated administration of antidepressant drugs in bulbectomised rats and suggest that these drugs reverse many or all of the behavioural changes produced by the lesion (Cairncross *et al*, 1978; Van Reizen *et al*, 1977). It appears that the mechanism of action of the antidepressant drug is not an issue for attenuation of the hyperactive response of the OB rat as the drugs used in this study act via different mechanisms to alleviate clinical symptoms. The newer antidepressants venlafaxine and milnacipran were also found to reverse the OB-induced hyperactivity thus confirming the model's status as a 'screening test' for compounds that display antidepressant activity in patients. All of the antidepressant drugs in this investigation were also observed to attenuate rearing scores in the open field however, only venlafaxine significantly attenuated measures of this parameter.

Previous studies (McGarvey, 1996) have shown no significant differences between sham-operated and un-operated (naïve) controls in behavioural tests including the open field test. In these investigations, SO and un-operated animals displayed a similar response to open field testing, amongst others suggesting that sham operation does not result in a significantly altered "control" animal and that an un-operated control group

in such studies is unnecessary. These results formed the basis of Study 2, which investigated exploratory activity in the open field over 4 weeks in SO animals only.

Study 2 demonstrated reduced ambulation scores in the open field over 28 days in vehicle and antidepressant treated SO animals. These effects are most likely to be due to a habituation response, as observed in control animals, being developed in the SO groups following repeated exposure to the open field arena. Similar effects were observed on rearing, measures for which were generally higher in antidepressant groups suggesting that antidepressant administration may be facilitating rearing activity in some way.

4.4.3. Effects of olfactory bulbectomy and antidepressant administration on specific ^{3}H-dexamethasone binding to hippocampal CR.

The antidepressant compounds used in these studies did not significantly affect specific ^{3}H-dexamethasone binding to CR in brain tissue (as observed in the competition experiments, section 4.3.1). Therefore any changes observed in CR binding parameters cannot be attributed to residual drug effects.

Despite the numerous correlates in disturbed endocrinological activity observed between human depressed patients and the OB rat, it appears that the dysfunctional corticosteroid feedback, characterised by a down-regulation of corticosteroid receptors in patients is not reflected in this animal model. The preceding experiments in Study 1 showed no reduction in specific ^{3}H-dexamethasone binding to CR in hippocampus from bulbectomised rats and B_{max} values were found to be very similar to those in SO animals.

The lack of a CR deficit in the OB rat was unexpected as previous studies have reported increased corticosterone output in this model, reversible by chronic antidepressant treatment (Catterelli & Damael, 1986). The ability of the OB rat to mount an additional corticosterone response to superimposed stress has also been reported (Cairncross *et al*, 1977; 1979) suggesting that the bulbectomised rat is chronically stressed, like the hypercortisolaemic depressed patient. However, there was quite clearly no CR deficit in the OB rat in our studies.

Although rats were sacrificed at approximately the same time of morning as in the naïve studies, they were subjected to a stressor (the open-field) prior to being killed. Some studies have reported increased and prolonged corticosterone secretion in bulbectomised rats, as compared to sham controls, subjected to the open-field (Kelly *et al*, 1995) and the additional response to stressors has already been mentioned.

Though a prolonged corticosteroid response to stress as implied in the OB rat, results in desensitised CR, frequent handling of animals produces the opposite effect resulting in suppression of fearful behaviour, up-regulation of GR and rapid termination of the stress response. Meaney *et al* (1989) showed that frequent handling of rats could increase GR binding capacity in the hippocampus of adult animals by 50%. Frequent handling was an integral part of the OB protocol and needed to be conducted to abolish the fearful and aggressive behaviour that would otherwise arise in bulbectomised rats (Leonard & Tuite, 1981). This may have confounded the investigation, as it is possible that the OB procedure and the handling procedure may act in opposite directions on CR and HPA-axis function.

Olfactory bulbectomy affects circadian rhythms by an unknown mechanism (Vagell *et al*, 1991) therefore disruptions in rhythmicity of corticosterone secretion may contribute to inconsistent HPA function.

The extensive degeneration of innervations to various brain regions that follows olfactory bulbectomy is well-documented (see section 4.1). Also well documented is the control of HPA-axis function by various neurotransmitters and neuropeptides (Dinan, 1994; 1996b). It is very clear that the widespread disruptions in neuronal functioning following bulb ablation would affect regulation of the HPA-axis in a number of ways (Cairncross *et al*, 1977; Dinan, 1994; Marcilhac *et al*, 1999). For example, 5-HT plays an important role in the bulbectomy syndrome exerting effects on aggressive, explorative and motivated behaviour. 5-HT is also intimately involved with the release of ACTH and corticosterone as 5-HT precursors, reuptake inhibitors and receptor agonists are all found to stimulate ACTH and corticosterone secretion (section 1.4.3). The 5-HT/HPA-axis loop appears to be anatomically and physiologically complex with multiple sites of interaction, one of these being the amygdala which has close connections with the limbic system (the corticomedial area of the amygdala inhibits ACTH secretion (Gloor *et al*, 1976) and is also a major site of 5-HT activity. It is also reported that the amygdala is heavily affected by bilateral olfactory bulbectomy (see section 1.6.1) thus affecting modulation of both 5-HT and HPA systems, which in turn appear to regulate each other. The co-localisation of GR and 5-HT_{1A} receptors in hippocampus may also support this (Dinan, 1996; Lopez *et al*, 1998). Interactions between all the factors involved are extremely complex and appear also to be multidimensional therefore the lack of impaired negative feedback of corticosteroids on hippocampal CR (via CR down-regulation) in the OB rat may be due to a number of factors. At this point, potential region specific effects must also be considered as it is

possible that an impairment of negative feedback may exist in other regions such as the hypothalamus or pituitary however CR binding in these regions was not measured therefore cannot be commented upon.

The effects of citalopram, venlafaxine, DMI and milnacipran administration were not strikingly different between OB and sham groups. Chronic administration of DMI and milnacipran did not produce any significant effects on the specific ^{3}H-dexamethasone binding to hippocampal CR in sham/OB rats. Following 28 days of administration, increases in B_{max} of specific ^{3}H-dexamethasone binding to CR of 26% and 30% were observed for citalopram and venlafaxine respectively. The observed increase in B_{max} following chronic citalopram administration is inconsistent with our own studies in hippocampus (from naïve animals) using a similar antidepressant, paroxetine (see sections 3.4 and 3.5). The results of previous studies using other SSRI's have also showed no effects of these drugs on hippocampal CR/CR mRNA following long term administration (Seckl & Fink, 1992; Budziszweska *et al*, 1994a; 1994b). Venlafaxine administration also had no effects on CR binding in our previous studies (see sections 3.4 and 3.5) therefore the increase in B_{max} observed in this investigation does not correspond with earlier observations. However, all of these investigations were conducted in naïve animals therefore comparisons with the OB investigations are difficult to make.

Due to the lack of a CR deficit in the OB rat model, it was decided to investigate the time course of antidepressant action on hippocampal CR in SO rats only. As mentioned previously, it appears that SO and un-operated animals are very similar in their behavioural responses. An extrapolation of this finding would suggest that sham-operation results in a neurochemically and endocrinologically unaltered "control"

animal therefore making an un-operated, naïve group redundant in such studies. In that the sham-operated control is similar to the naïve animal, it can therefore be compared to the investigations in chapter 3.

The antidepressant compounds used in these studies had no significant effects on the B_{max} of specific ^{3}H-dexamethasone binding to hippocampal CR following chronic administration with the exception of venlafaxine which increased B_{max} values by 30% at day 28.

To compare citalopram administration in this study broadly with paroxetine administration in the naïve study (as both drugs belong to the same group of antidepressants, SSRI's), it was found that chronic citalopram administration had no significant effects on hippocampal CR in this study. Budziszweska *et al* (1994a) found no effect of citalopram on hippocampal CR. Seckl & Fink (1992) also reported no effect of citalopram on GR mRNA in rat hippocampus. In the naïve study, no significant effects of paroxetine treatment were observed in the hippocampus, which generally agrees with the present study. However, a down-regulation of CR (in cortex and thymus) following paroxetine treatment is observed implying the presence of different mechanisms in different regions.

Venlafaxine administration had no effects in the naïve animals used in studies conducted in Chapter 3. In the SO animals, venlafaxine administration for 28 days induced a significant increase in B_{max} values, with a corresponding increase in K_D values at this time point indicating potentially different mechanisms of this compound in naïve and SO animals.

Due to the differences following antidepressant administration in SO and naïve animals, it may be that SO rats are not as similar biochemically to naïve animals used in chapter 3. Many factors may have complicated this study and the animals were treated quite differently in both studies including differences in doses of antidepressants given and routes of administration.

The olfactory bulbectomised rat appears to be an attractive model in which to investigate the neurobiological basis of depression and the mechanism of action of antidepressant drugs. Many neurochemical and neuroendocrine mechanisms have been associated with the behavioural and physiological aspects of the OB syndrome, a large proportion of which display impressive similarities to the clinical symptomatology. However, it appears that the OB rat does not model the impairment of negative feedback (thought to result from the down-regulation of corticosteroid receptors) observed in depressed patients and the restoration of HPA feedback activity (resulting from CR up-regulation) following chronic antidepressant treatment.

CHAPTER 5

PREDATOR STRESS STUDIES; DEVELOPMENT & OPTIMISATION OF BEHAVIOURAL TESTING PROTOCOLS

5.1. Introduction

Several animal models of depression attempt to parallel the putative role of stress in producing depressive states (Sherman & Petty, 1984; Katz *et al*, 1981). Alterations in behaviours such as locomotor activity and anhedonia represent core symptoms of depression and are also relatively easy to measure in laboratory animals however, behavioural testing can be highly subjective and variable between investigations and research laboratories. This chapter describes the basic developmental and optimisation procedures for behavioural tests conducted in our laboratory that were applied in later predator stress studies (see Chapter 6). Modifications were also made to the CR binding assay in order to adapt it for the smaller mouse tissues.

The mouse open field arena used in this, and subsequent chapters, was based on the rat open field arena used in chapter 4. Modifications were made to dimensions and markings of the arena on consultation with previous literature (Logue *et al*, 1997) and through personal communications (Karen Mellowdew - Institute of Psychiatry- see section 5.3). It was decided to use this test in future predator stress investigations in order to allow for some degree of continuity and comparison between the OB rat investigations and the predator stress studies.

Some studies have suggested that locomotor activity may be best measured during the dark phase of the light: dark cycle as rodents are nocturnal animals and measurements taken at this time would more accurately reflect normal activity levels (Batchelor, 1994; Calamandrei *et al*, 1997). Circadian fluctuation may also affect locomotor activity in the open-field arena. The effects of both of these factors were investigated in this chapter. The period of open field testing also shows great variation across many different laboratories with testing times between 3 and 20 minutes per animal (de

Angelis, 1996; Calamandrei *et al*, 1997). Open field measures were also taken over various testing times to establish an efficient and accurate test period.

The development of sucrose testing as a measure of anhedonia and decreased reward sensitivity was initially used by Willner *et al* in 1987(a). Since then, the protocol for sucrose testing has undergone numerous modifications and is found to display enormous variation between research groups.

The concentration of the sucrose solution (the reward substance) is very important in measuring anhedonic behaviour using sucrose tests. This is dictated by the fact that the sucrose concentration-intake function is bell-shaped (Muscat & Willner, 1989; Phillips *et al*, 1991b; Monleon *et al*, 1995). A sucrose concentration gradient was established prior to testing in order that an appropriate sucrose concentration could be chosen for future studies.

Sucrose tests can be conducted using 1 bottle (consumption) or 2-bottle (preference) tests and differences have been found in the relationship between sucrose concentration and reward sensitivity using both of these types of tests (Phillips *et al*, 1991a; Muscat & Willner, 1992). Sucrose test periods are also found to differ between laboratories therefore an accurate and efficient test period was established by investigating intake over various test times.

As suggested for tests measuring locomotor activity, the implication for sucrose testing is also that it may be more appropriate to conduct these tests in the dark phase of the animals activity cycle. The effect of testing in light or dark phases of the activity cycle was also investigated in this chapter using reversed light: dark cycles.

The issue of food and water restriction/deprivation prior to sucrose testing has been a controversial one (Muscat & Willner, 1992a; Willner *et al*, 1996; Harris *et al*, 1998; Forbes *et al*, 1996). Sucrose intake was established with and without prior food and water deprivation to ascertain the effects, if any, on intake volumes.

As in initial experiments that aimed to optimise the CR binding assay for rat cytosolic preparations (see section 2.6), it was also necessary to establish appropriate mouse tissue concentrations in CR binding assays for future investigations in this chapter.

As the following investigations (Chapter 6) involved the development of a potential "predator stress" paradigm in mice, many preliminary investigations were undertaken prior to beginning of the stress studies. The main purpose of this chapter was to establish optimum behavioural test and assay conditions in our laboratory. Methods used in the preliminary investigations are initially presented with the data generated, followed by the standard procedures adopted as a consequence of these preliminary experiments.

5.2. Animals

Male adult BALB/c mice from the NESCOT breeding stock were used in these investigations. Animals were 12-14 weeks old and weighed 20-25 g at the start of the studies. Mice were individually housed beginning one week before the start of the experiments. All animals were maintained on 12:12 hour light:dark cycle (lights on 06.00-18.00hrs) and were allowed free access to standard laboratory mouse food and water except as described in sections 5.3.1.iii, 5.5.1.ii and 5.5.1.iii (open field / sucrose testing under reversed light: dark cycles and following food and water deprivation).

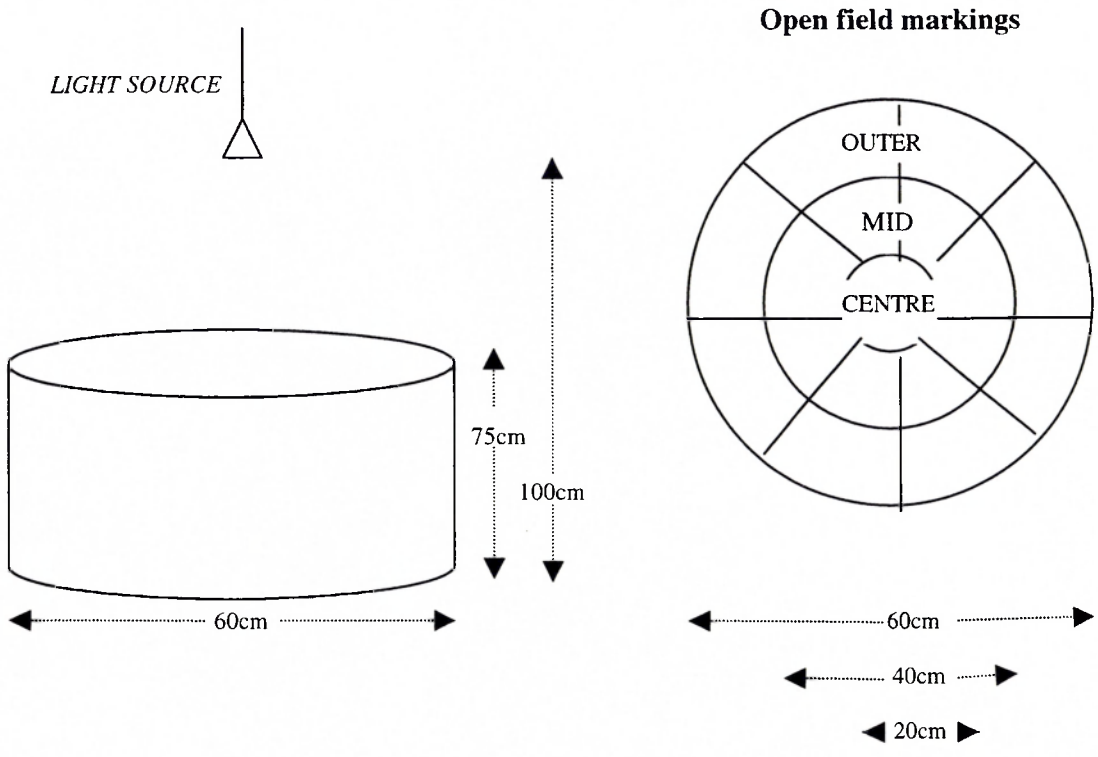
5.3. Optimisation of open field testing protocol

The aim of these preliminary experiments was to establish optimum conditions in our laboratory, under which to observe the open-field behaviour of BALB/c mice in order to obtain an efficient and realistic measure of locomotor activity.

5.3.1. Methods

Locomotor activity was determined using a circular open-field arena (diameter of 60cm) made of vinyl with a white base subdivided by yellow lines into centre, mid and outer circles which were each further divided into smaller segments (Figure 5.3.1). The inside surface of the arena was coated with reflective aluminium paint and the arena was illuminated directly from above using a 100-watt bulb. Individual mice were transferred from the home cage to the open field arena. The test was started by placing the animal in the centre of the open-field arena. The behaviour of the mice was then observed and video recorded for various periods of time. Locomotor activity of each animal was observed by means of a television monitor in a separate room in order to minimise the amount of external stress experienced by the animals. All measurements were taken in a darkened room.

Figure 5.3.1. Mouse open-field arena



The following behaviours were recorded in the open-field arena:

a) *ambulation* : the number of crossings of lines with both forepaws.

b) *grooming* : the number of times the mouse stopped and groomed itself.

c) *rearing* : the number of times the mouse simultaneously raised both forepaws off the floor of the apparatus.

d) *defecation* : the number of faecal boli deposited.

e) *mobility latency* : time taken from 0 mins for the mouse to start moving within the apparatus.

f) *orientation* : the portion of the apparatus in which the mouse spent the most amount of time.

The open-field arena was cleaned with 3% alcohol and dried after each test.

The following open-field investigations were conducted in order to ascertain a protocol for future studies;

i) Determination of open-field activity of BALB/c mice during light:dark phase of the diurnal activity cycle. Open-field activity was measured in animals that were housed under normal (lights on 06.00–18.00 hrs) and reversed (lights on 18.00–06.00 hrs) light cycles in order to ascertain diurnal variations of activity levels. Animals were placed in the open field arena for a 5-minute period (between 09.00-11.00 hrs) and the activity recorded as described above. Differences in activity during the light or dark phase were determined by subjecting the data to t-tests/Mann-Whitney tests (GraphPad Prism 3.0).

ii) Determination of open-field activity of BALB/c mice at different times during the day. Activity in the open-field arena was observed at different times of day to determine the effects of circadian fluctuations on the locomotor activity of BALB/c mice housed under normal light:dark cycles. Open-field behaviour was recorded over a 5-minute period in the morning (between 09.00-11.00 hrs) or the afternoon (between 13.00-15.00 hrs). Experiments were conducted twice and data pooled, with differences being determined by t-tests or Mann-Whitney tests (using GraphPad Prism 3.0).

iii) Open-field activity of BALB/c mice over different test periods. Locomotor activity of BALB/c mice was measured over different periods of time (3, 5 and 10 minutes) to allow for comparison of the observations made over these periods and determination of a suitable time "window" during which to measure open-field activity. This experiment was conducted four times using animals housed under normal light:dark cycles, the data pooled and analysed using one-way ANOVA or Kruskal-Wallis test (using GraphPad Prism 3.0).

5.3.2. Results

i) Determination of open-field activity of BALB/c mice during light:dark phase of the diurnal activity cycle. Ambulation and defecation scores conformed to a normal distribution therefore were analysed using t-tests. Grooming and rearing scores did not conform to a normal distribution and were consequently analysed using the non-parametric Mann-Whitney test.

Ambulation scores of BALB/C mice in the open-field were not significantly different when measured in the light or dark phase (see Table 5.3.2.i.). Grooming scores were significantly lower in animals tested during the dark phase however, there were no significant differences in rearing and defecation counts from animals tested in the light or dark phase. There were no significant differences in the orientation within the open field arena of mice tested in the light or dark phases.

Table 5.3.2.i. Open-field activity of BALB/c mice in light/dark phase of the diurnal cycle.

PARAMETER	LIGHT PHASE	DARK PHASE
Ambulation	32 ± 7	45 ± 8
Grooming	0.88 ± 0.18	0.33 ± 0.13 *
Rearing	0.38 ± 0.38	0.07 ± 0.07
Defecation	4.6 ± 0.4	6.1 ± 0.8
Orientation	outer	mid / outer

Open-field activity of BALB/c mice was measured as described in section 5.3.1.i. Data are expressed as mean ± sem (n=15-16) and were subjected to t-tests (ambulation and defecation counts) and Mann-Whitney tests (grooming and rearing counts) using GraphPad Prism 3.0. Statistically significant differences are denoted by * $p<0.05$.

ii) Determination of open-field activity of BALB/c mice at different times during the day. There were no significant differences in ambulation scores of animals tested in the morning or afternoon (see Table 5.3.2.ii). Grooming counts were significantly higher in mice tested in the afternoon as compared to those tested in the morning. No differences were observed between rearing scores measured in the morning or afternoon. Defecation counts were significantly higher when measured in the morning as compared to afternoon values. There were no significant differences in the proportion of time spent by the animals in the various regions of the open-field arena at different times of testing.

Table 5.3.2.ii. Open-field activity of BALB/c mice tested at different times of day (AM/PM).

PARAMETER	MORNING	AFTERNOON
Ambulation	52 ± 9	49 ± 9
Grooming	0.56 ± 0.18	1.78 ± 0.35 *
Rearing	0.38 ± 0.38	0.7 ± 0.27
Defecation	4.2 ± 0.5 *	2.9 ± 0.5
Orientation	mid / outer	outer

Open field activity of BALB/c mice (housed under a normal light:dark cycle) was measured as described in section 5.3.1.ii. Data are expressed as mean ± sem (n=16-18) and were subjected to t-tests (ambulation and defecation counts) and Mann-Whitney tests (grooming and rearing counts) using GraphPad Prism 3.0. Significant differences are denoted by * $p<0.05$.

iii) Open-field activity of BALB/c mice over different test periods. All activity measures were significantly increased with length of test period therefore statistical differences are not displayed (see Table 5.3.2.iii). Variability of these measures was highest with the shortest test period. Standard errors were approximately 15% of the mean for 3 minute test periods and approximately 10% of the mean for 5 and 10 minute test periods. Animals spent the majority of the test in the mid/outer region of the open-field arena whether the testing time was 3, 5 or 10 minutes.

Table 5.3.2.iii. Open-field activity of BALB/c mice recorded over different test periods

PARAMETER	3 min test	5 min test	10 min test
Ambulation	30 ± 4	50 ± 6	76 ± 8
Grooming	0.59 ± 0.15	1.21 ± 0.23	2.0 ± 0.38
Rearing	0.06 ± 0.04	0.5 ± 0.22	0.88 ± 0.39
Defecation	2.2 ± 0.2	3.6 ± 0.4	7.4 ± 0.6
Orientation	mid / outer	mid / outer	mid / outer

The activity of BALB/c mice, housed under a normal light:dark cycle, was measured over periods of 3, 5 (n=34, over several different experiments) and 10 minutes (n=8, over two different experiments) as described in section 5.3.1.iii. Data are expressed as mean ± sem and were subjected to one-way ANOVA (ambulation and defecation counts) and Kruskal-Wallis tests (grooming and rearing counts) using GraphPad Prism v3.0. All groups were significantly different from each other therefore no symbols denoting statistical significance are shown. Statistically significant differences of measured parameters between each test period ($p<0.001$ - ambulation, $p<0.01$ - grooming, $p<0.01$ - rearing, $p<0.0001$ - defecation).

5.3.3. Discussion

These investigations aimed to establish an optimal open-field test (OFT) protocol for BALB/c mice in our laboratory. Following the testing of BALB/c mice in both the light and dark phases of their cycles, it was found that most of the measures obtained in the OFT were not affected by the light phase of the animal. The nocturnality of mice is well documented and it has been suggested that, in order to gain a true measure of activity in nocturnal animals, testing should be carried out in the dark phase of the light:dark cycle (de Angelis *et al*, 1996) however, we found no differences in measures taken in the light or dark phase. Open-field testing in future studies was conducted during the light phase of the mice, as this was more convenient and avoided disruption of the animal holding facilities.

Following a decision to perform open-field testing in the light phase of the BALB/c mice, we tested the effects of circadian rhythms on OFT measures by comparing data from tests conducted in the morning and afternoon. Again, many of the measures taken were not significantly affected by the time of day. On the basis of these data, it was decided to conduct subsequent OFTs at a consistent time in the morning (08.00-11.00hrs) in order to avoid the possible influences of circadian rhythmicity.

In order to establish our OFT protocol, we conducted experiments in which BALB/c mice were subjected to OFT for periods of 3, 5 and 10 minutes. Three minute test periods were too short as the counts obtained were not high enough to allow accurate measurement of possible alterations following other experimental procedures. This group also displayed larger standard errors. Open-field activity measured over 5 and 10 minutes gave measurable counts and similar values for standard errors. As the 10

minute test was rather time-consuming and laborious, open-field testing was conducted over a 5 minute period in future investigations.

Locomotor activity is found to have a strong genetic influence and studies correlating strain of mice with activity have demonstrated enormous quantitative differences in measures of locomotor activity across genotypes (Logue *et al*, 1997). In an assessment of locomotor activity in various mouse strains using the open field arena, mean ambulation scores in BALB/c mice were quite low (~80 over a 5 minute test period) with locomotor activity being divided almost equally over the first and second 2.5 minutes of the test. Defecation scores in the BALB/c strain was the highest (~8 over the 5 minute test period) of all strains tested. As BALB/c mice are an albino/visually impaired strain, these researchers have suggested that the gene for albinism may have an impact on locomotor activity (but is not an absolute predictor of low activity). This may explain the generally low activity levels observed in our mice.

Open field measures are also found to be influenced by the type of arena used to record activity. This can have a very large effect on the type of behaviour displayed by the animal (Lister *et al*, 1990). Individual housing has also been proposed as a factor that may influence activity – this is further discussed in chapter 6.

5.4. Determination of optimal concentration for sucrose tests

This phase of the investigations consisted of i) ascertaining any preference of BALB/c mice for sucrose solutions over water and ii) choosing an appropriate sucrose concentration to be used in later sucrose intake experiments.

5.4.1. Methods

It was not clear at this stage whether sucrose consumption (1-bottle) or preference (2-bottle) tests would be utilised in later studies therefore a sucrose gradient was determined using both types of sucrose intake tests.

i) Determination of a sucrose gradient using sucrose preference (2-bottle) test. Forty-eight mice were divided into 8 groups (n=6) and their intake of both water and sucrose solutions at various concentrations (0%, 0.5%, 1%, 2%, 4%, 8%, 16% and 32%) was measured. Two bottles, one containing water and the other containing one of the above sucrose concentrations were presented per cage (in a random manner) over 24 hours and intake was measured by weighing the bottles before and after the test period.

ii) Determination of sucrose gradient using sucrose consumption (1-bottle) tests

Forty-eight mice were divided into 8 groups (n=6) and tested for their intake of sucrose solutions at various concentrations (0%, 0.5%, 1%, 2%, 4%, 8%, 16% and 32%) over 24 hours when a bottle containing one of the above sucrose concentrations was given (in a randomised position), in place of water. The intake of sucrose was measured by weighing the bottle containing the solution before and after the test period.

Sucrose and water intake values were corrected for leakage from the test bottles, which had been previously determined from a number of experiments. Corrected values were used in the analysis of experiments.

5.4.2. Results

i) Determination of a sucrose gradient using sucrose preference (2-bottle) tests. The mean sucrose and water intakes over the 24 hour testing period using the 2-bottle test are shown in Figure 5.4.2.i. Sucrose intake generally increased as a function of sucrose concentration up to a concentration of 16% but decreased at higher concentrations. Water intake was much lower than sucrose intake with the greatest difference at a sucrose concentration of 16%.

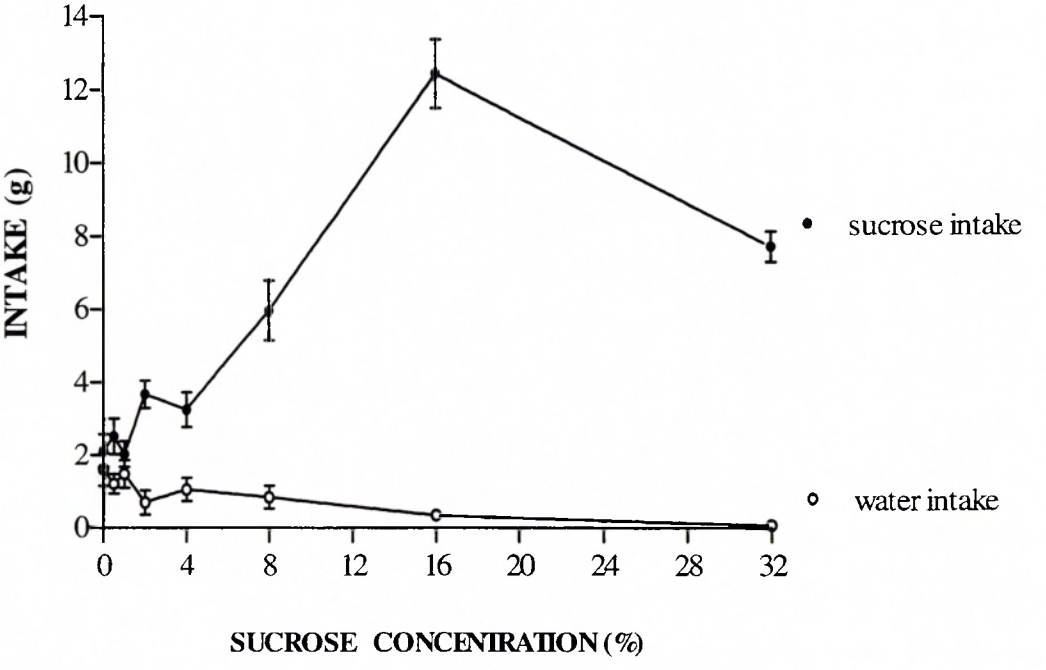


Figure 5.4.2.i. Intake of water and different concentrations of sucrose (0%, 0.5%, 1%, 2%, 4%, 8%, 16% and 32%) over 24 hours using the 2-bottle intake test in BALB/c mice. Results are expressed as mean ± sem (n=6 per concentration).

ii) Determination of sucrose gradient using sucrose consumption (1-bottle) tests. Mean sucrose intake over the 24 hour testing period is shown in Figure 5.4.2.ii. Intake generally increased up to sucrose concentrations of 16% but decreased at higher concentrations.

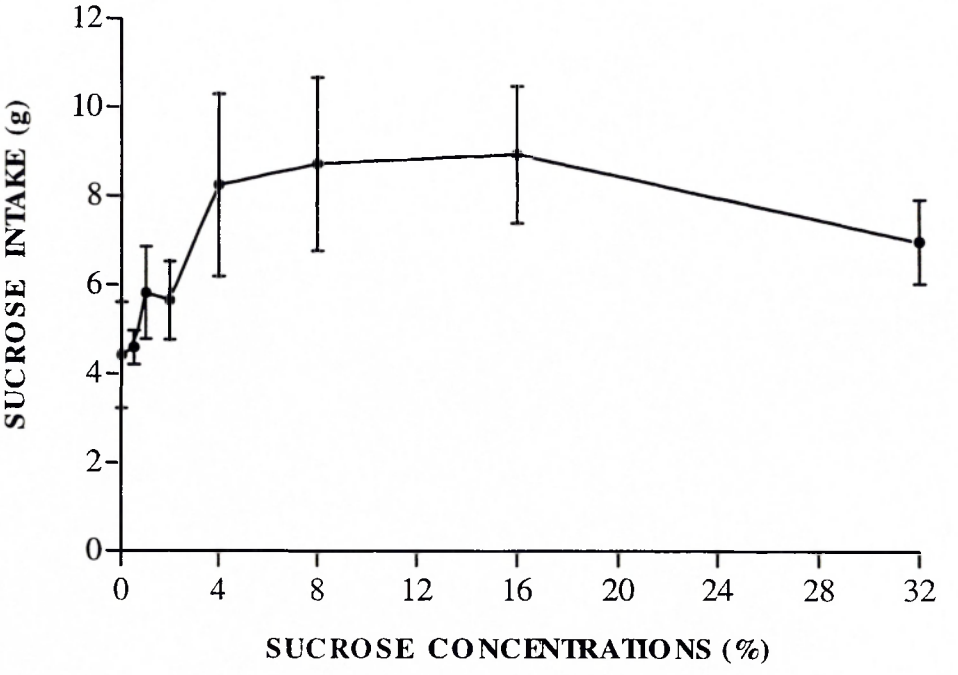


Figure 5.4.2.ii. Consumption of different concentrations of sucrose (0%, 0.5%, 1%, 2%, 4%, 8%, 16% and 32%) over 24 hours using the 1-bottle intake test in BALB/c mice. Results are expressed as mean ± sem (n=6 per concentration).

5.4.3. Discussion

These experiments aimed to establish an appropriate sucrose concentration for use in future experiments. Sucrose intake in our experiments followed an inverted U-shaped function in both preference and consumption studies however this observation was more pronounced in the preference experiments. Data obtained in our sucrose gradient experiments were consistent with those of Towell *et al* (1987), Muscat & Willner (1989) and Phillips *et al* (1991a; 1991b).

The sucrose concentration-intake function has previously been demonstrated as being bell-shaped in sucrose gradient experiments conducted in rats (Muscat & Willner, 1989; Phillips *et al*, 1991a; 1991b) and in mice (Monleon *et al*, 1995). At low concentrations, on the ascending limb of the concentration-intake function, sucrose

intake rises monotonically with concentration and is related to reward value (as assessed by preference measures in choice tests). However, at high concentrations, on the descending limb of the concentration-intake function, sucrose intake is no longer related in a simple way to reward value yet cannot be associated with aversive or satiety effects (Towell *et al*, 1987; Muscat & Willner, 1989; Phillips *et al*, 1991a; 1991b).

On the basis of these experiments, a sucrose concentration of 3% was chosen for subsequent experiments as it was thought that this would allow for accurate measurement of alterations in sucrose intake in later studies therefore would reflect changes in the reward value of the sucrose solution. Previous sucrose intake experiments in mice have used 2% sucrose solutions (Monleon *et al*, 1995; D'Aquila *et al*, 1997a; 1997b). Sucrose concentrations of 6-12% displayed the highest intake therefore would not be likely to reveal any increases in intake. At the higher concentrations of sucrose solutions, intake was observed to decrease as in the aforementioned studies.

5.5.Determination of sucrose intake testing protocol

5.5.1. Methods

The aim of these preliminary experiments was to determine the sucrose intake of BALB/c mice using either 2-bottle preference or 1-bottle consumption tests. Sucrose intake was measured by weighing the bottles before and after the test period following which, values were corrected for leakage. The following experiments were conducted in order to establish a standard protocol for sucrose testing in BALB/c mice.

i) Sucrose tests over 1 and 3 hour periods. Sucrose intake testing was initially conducted over periods of 1 and 3 hours in order to ascertain intake volumes that would reflect changes in future studies. BALB/c mice, housed under a normal light:dark cycle, were tested for intake of water or 3% sucrose solutions over these periods using both 1 and 2 bottle tests.

ii) Sucrose tests following food and water deprivation. Sucrose intake tests (1 bottle) were conducted in animals subjected to 3 hours of food and water deprivation and also in mice that had not been deprived in order to ascertain any differences between the groups. All mice were housed under a normal light:dark cycle.

iii) Sucrose tests at different times of the light:dark activity cycle. Sucrose intake tests (1 bottle) were carried out in BALB/c mice which had been housed under normal (06.00-18.00hrs) and reversed (18.00-06.00hrs) light:dark cycles to ascertain any differences in intake at various times during the activity cycle.

Experiments were repeated in several groups of mice, the data pooled and subjected to t-tests (using GraphPad Prism v3.0) in order to determine any significant differences between the groups.

5.5.2. Results

i) Sucrose tests over 1 and 3 hour periods. BALB/c mice demonstrated a clear preference for the 3% sucrose solution over water in both the 1 and 3 hour 2-bottle preference tests (see Table 5.5.2.i.). The intake of a 3% sucrose solution in BALB/c mice over 1 hour and 3 hours in 1-bottle consumption tests was not significantly different although 3 hour tests did provide higher volumes of sucrose intake. However, longer testing times yielded increased intake in 2-bottle preference tests as these values differed for both water and 3% sucrose over 1 and 3 hour tests. Sucrose intake over a 1 hour test period was significantly higher in the 1-bottle test as compared to that in the 2-bottle test. Over a 3 hour test period however, sucrose intake values obtained using 1-bottle tests were not different from those obtained using 2-bottle tests.

Table 5.5.2.i. Sucrose and water intake in BALB/c mice using 1 and 2 bottle tests over 1 and 3 hour test periods.

test time	**1-BOTTLE TEST**	**2-BOTTLE TEST**	
	sucrose intake (g)	water intake (g)	sucrose intake (g)
1 hour	1.06 ± 0.24	0.41 ± 0.06	0.63 ± 0.06 *Δ
3 hours	1.21 ± 0.1	0.8 ± 0.13 #	1.16 ± 0.11 *#

Sucrose and water intake of BALB/c mice (n=12 for 1-bottle test over 1 hour; n=20-21 for all other groups, across several different experiments) was tested over 1 and 3 hour periods as described in section 5.5.1.i. Data are expressed as mean ± sem. Statistically significant differences were determined using t-tests (GraphPad Prism v3.0) and are denoted by; * $p<0.05$ - as compared to water intakes in 2-bottle tests over 1 and 3 hours, # $p<0.05$ - as compared to 1 hour test intake values in 2-bottle tests and Δ $p<0.05$ - as compared to the 1-bottle sucrose intake values over the same test period.

ii) Sucrose tests following food and water deprivation.

Animals that had been deprived of food and water for 3 hours prior to testing displayed a significantly higher sucrose intake than non-deprived mice (see Table 5.5.2.ii). Variability was similar in both groups of animals.

Table 5.5.2.ii. Sucrose intake in food and water deprived and non-deprived BALB/c mice

	food + water deprived	non-deprived
sucrose intake (g)	1.08 ± 0.05	0.80 ± 0.06 *

Sucrose intake tests in i) non-deprived mice and ii) mice deprived of food and water for 3 hours (n=60 for each group, across several experiments). Tests were conducted as described in section 5.5.1.ii. Data are expressed as mean ± sem. Statistically significant differences were determined using t-tests (GraphPad Prism v3.0) and are denoted by; * $p<0.05$.

iii) Sucrose tests at different times of the light:dark activity cycle. Though mice tested in the dark phase appeared to consume more of the 3% sucrose solution than those tested in the light phase, there were no statistically significant differences in sucrose intake of mice during the light or dark phases of their activity cycles (Table 5.5.2.iii)

Table 5.5.2.iii. Sucrose intake in BALB/c mice in the light and dark phase of the activity cycle.

	light phase	dark phase
sucrose intake (g)	1.06 ± 0.24	1.32 ± 0.2

Sucrose intake in BALB/c mice (n=12 across several experiments) using 1-bottle tests i) in the light phase ii) in the dark phase of the activity cycle. Tests were conducted as described in section 5.5.1.iii. Data are expressed as mean ± sem. No statistically significant differences were determined using t-tests (GraphPad Prism v3.0).

5.5.3. Discussion

BALB/c mice displayed a clear preference for the 3% sucrose solution over water in 2-bottle preference tests suggesting that the intake of this solution may constitute a reward stimulus. The test period did affect the intake volume and, as we were attempting to maximise these, it was decided to use 3-hour test periods in all future experiments. As there were no significant differences between sucrose intakes over 3 hour periods using 1 or 2 bottle tests, it was decided to use 1 bottle consumption tests in future studies as these were more conveniently conducted. The relationship between sucrose concentration and intake using 1-bottle tests is also thought to be more robust than that using 2-bottle sucrose tests (Muscat & Willner, 1992b).

In our experiments, intake volumes were significantly higher in the group that had been deprived of food and water prior to sucrose testing. No differences were observed in variability between the deprived and non-deprived groups. Therefore it was decided to subject animals to a 3-hour deprivation period prior to sucrose testing in future studies. This is a controversial issue as several studies have suggested that decreased intake of sucrose following chronic, unpredictable, mild stress (CMS) in food and water deprived laboratory animals may be related to loss of body weight and calorie intake (Forbes *et al*, 1996), or dependant on their dehydration state (Harris *et al*, 1998). However, other studies have also demonstrated that sucrose consumption in not secondary to loss of body weight (Willner *et al*, 1996) and unrelated to calorie content (Muscat *et al*, 1991; see section 6.3.4).

Mice tested in our experiments showed no difference in sucrose consumption whether tested in the light or dark phase. Consequently, it was decided to conduct all sucrose

testing in the light phase of the mouse activity cycle, as this was more convenient and also would avoid disruption of the animal holding facilities.

Previous studies in which 2% sucrose solutions were used in order to measure anhedonia following CMS in mice have demonstrated considerable deficits in sucrose consumption following stress. Our tests using 3% sucrose were consistent with these and ensured that the sucrose concentration being utilised was on the ascending limb of the concentration-intake function.

Recent investigations have identified a genetic basis of preference for sweet substances among inbred strains of mice (Capeless & Whitney, 1995). The BALB/c strain used in our studies is thought to have preference for sodium saccharin at high concentrations (2-bottle, 48 hour preference test) due to the involvement of the genetic loci, *Sac* and *dpa*, suggested as important factors in the observed variation among inbred mouse strains for response to sweet substances (see section 6.3.4).

5.6. Determination of a linear relationship between specific binding and concentration of mouse cortical cytosol

These experiments were conducted to demonstrate the presence of artefacts if any, in the binding assay and also to ensure that an appropriate tissue concentration was utilised in the assay to yield measurable specific binding values (see section 2.6). The smaller size of mouse brain regions also made it necessary to repeat these experiments as the concentration of mouse tissue required may have differed considerably from that used in binding assays using rat tissue.

5.6.1. Methods

Mouse cortical cytosol fractions were prepared as described in section 2.3 except that cortical tissue was homogenised in 1:10 w/v of incubation buffer and following centrifugation, the tissue supernatant was diluted to the required concentration. 100µL aliquots of the tissue supernatant were incubated with either 10% ethanol or 5µM hydrocortisone and 3nM ^{3}H-dexamethasone and subsequent procedures were carried out as described in section 2.3.

The specific binding of ^{3}H-dexamethasone to CR in the cytosol preparation was calculated by subtracting NSB from TB and plotted against tissue concentration. Linear regression analysis was performed on the data, using GraphPad Prism v.3.0, to determine the effects of tissue concentration on the specific binding of ^{3}H-dexamethasone.

5.6.2. Results

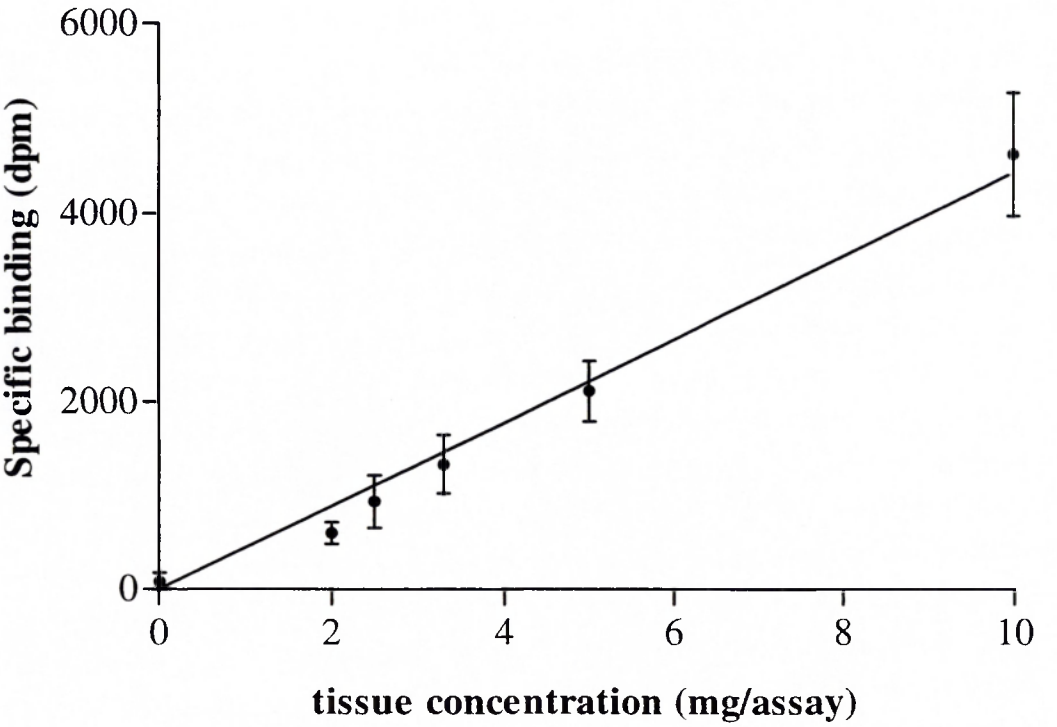
Specific binding of ^{3}H-dexamethasone increased linearly with increasing tissue concentration (see Figure 5.6.2.i). The values of specific binding observed at tissue concentrations of 5, 3.3 and 2.5 mg wet weight original tissue were 2115, 1334 and 937 dpm (corresponding to 12.7, 12.0 and 11.9 fmol/mg protein) respectively, thus giving measurable values for the specific binding of ^{3}H-dexamethasone at these concentrations of tissue. The mean values for added ^{3}H-dexamethasone in these assays was 171065 ± 4029 dpm and the amount of radioactivity bound as a percentage of added radioactivity was 1.4%.

5.6.3. Discussion

These investigations demonstrated that the use of the CR binding assay described in chapter 2 to measure the binding parameters of CR in mouse cytosolic preparation yields consistent results, which appear to be free of artefacts. A tissue concentration of 3.3mg wet weight original tissue was chosen from the linear portion of the graph (Figure 5.6.2.i.) and employed routinely in all subsequent binding experiments. This concentration of tissue did not bind more than 10% of the added radioligand in the assay and also gave measurable specific ^{3}H-dexamethasone binding to CR in mouse cytosolic fractions.

Figure 5.6.2.i.

Determination of the linear relationship between specific ^{3}H-dexamethasone binding and mouse cortical cytosol concentration



Mouse cytosolic fractions homogenised in 1:10 w/v of incubation buffer were prepared as described in section 5.6.1. The resulting supernatant was diluted to give cortical cytosolic concentrations of 2-10mg wet weight of tissue / incubation. Assay procedures were carried out as described in section 2.3. The data shown represents the mean (± sem) from five experiments.

5.7. Overall discussion

Our experimental results for each of the present studies do not vary greatly from those obtained in previous studies. It is not surprising that, with all the differences resulting from genetic, physiological and laboratory influences, that there should be so much variation in behavioural experiments and data. There are many factors that may affect behaviour patterns and we attempted as far as possible to control for these within our laboratory. Standard protocols were established following these investigations for each of the behavioural tests and also appropriate modifications made to the CR binding assay for mouse tissue.

Open field behaviour was assessed in future studies between 08.00-11.00 hours (during the light phase of the activity cycle) over a period of 5 minutes per animal. Sucrose intake testing in future studies was conducted as follows; animals were deprived of food and water for a period of 3 hours (09.00-12.00 hours) following which a single bottle containing a 3% sucrose solution was presented to them for a period of 3 hours (12.00-15.00 hours). Sucrose intake was calculated by weighing the bottles containing the solution before and after the test.

The use of the CR binding assay described in chapter 2 to measure specific binding of ^{3}H-dexamethasone to CR in mouse cytosolic preparations appears to yield consistent and accurate data. A tissue concentration of 3.3mg wet weight original tissue was employed routinely in all subsequent mouse CR binding experiments.

All behavioural testing and assay procedures were conducted as described above for subsequent predator stress studies presented in the following chapter.

CHAPTER 6

PREDATOR STRESS/ANTIDEPRESSANT STUDIES

6.1. Introduction

6.1.1. Predator stress as a model for stress/depression

Exposure of rats to the presence or odour of a cat constitutes a non-invasive, species-relevant, life threatening, inescapable stress that results in changes in affective functioning of the rat. Investigation of various behaviours measured during cat exposure suggest that these may reflect a phobic anxiety state (File, 1996a; 1996b). Several physiological, behavioural, immunological and neurochemical alterations have been reported in rats following exposure to cat odour suggesting that this predator stimulus may be a potent stressor.

There is some evidence to suggest that predator exposure may reduce growth in rats (Adamec *et al*, 1998). Predator exposure was also found to induce increases in blood glucose in male rats (Bialik *et al*, 1989); this effect was absent in animals that had their adrenal medullae surgically removed. Depletion of brain NA potentiated the increase in blood glucose observed following predator odour rather than impairing the blood glucose response.

The most commonly observed result of predator exposure is increased avoidance behaviour of responsive rats with little evidence of habituation following repeated exposure (File *et al*, 1993a; Zangrossi & File, 1994). Clear increases in avoidance behaviour have also been displayed in 7-day old chicks following exposure to predator odour (Fluck *et al*, 1996). Behavioural measures in the elevated plus maze have been investigated following predator exposures. Adamec & Shallow (1993a) demonstrated that a 5 minute exposure to a cat decreased activity/explorative behaviour for up to 3 weeks after exposure. Risk assessment behaviours as measured in the plus maze were also reduced by cat exposure indicating an enhanced state of anxiety in the stressed rats

(Adamec *et al*, 1993a). Zangrossi *et al* (1992a; 1992b) have also examined the after-effects of exposing rats to cat odour and found that anxiety-like behaviours were observed for some time after exposure. These investigations suggest that predator exposure may possess a long-lasting stress component.

The development of the anxiety/defence test battery to investigate defensive reactions of rats to a natural predator, the cat, have been very useful (Blanchard *et al*, 1990). Three paradigms have been used to study the effects of predator exposure; i) proxemics/activity, ii) eat/drink test and iii) risk assessment. In cat exposed rats, there was an increase in proxemic avoidance and decreased rearing and transits between sections. In the eat/drink test, there was a constant inhibition of non-defensive behaviour including reduced eating frequency and duration. Predator odour also induced increases in risk assessment and fear/anxiety behaviours including stretch-attend, flat back postures. The chronic administration of imipramine prior to testing reduced defensive stress-induced behaviours (Blanchard *et al*, 1993).

Numerous investigations have indicated that the various behaviours displayed by rats in the presence of a predator may relate to fear and those evoked by the odour of a predator suggest anxiety as the administration of anxiolytics is found to reverse some predator-induced effects (Adamec *et al*, 1993a; 1993b).

6.1.2. Neurochemical changes following predator exposure

Significant decreases in GABA enhancement of benzodiazepine binding have been observed in chicks following exposure to predator odour – changes associated with increased fear (Fluck *et al*, 1996). Hogg & File (1994) also demonstrated significantly lower benzodiazepine receptor binding in hippocampus and frontal cortex in responder

groups following predator stress. Increases in [^{14}C]-GABA release in both cortex and hippocampus, accompanied by decreases in uptake were observed in rat brain slices following cat odour exposure (File *et al*, 1993). Investigations conducted by Kavaliers & Colwell (1995) have also implicated the GABA system in predator-induced stress responses. Mice infected with a natural parasite were found to display reduced anxiety and spent more time in the vicinity of the cat odour as compared to non-infected mice. These altered predator responses were reduced by peripheral administration of the $GABA_A$ antagonists, bicuculline and picrotoxin indicating that parasite infection in mice reduces avoidance of predators at least partly though $GABA_A$ receptor mechanisms.

Changes in the release and uptake of 5-HT in rat brain slices following cat odour exposure were found to be markedly time dependent, with decreased hippocampal [^{3}H]-5-HT uptake and higher basal release in rats sacrificed immediately after stress. In rats killed 30 minutes after stress, there was increased ^{3}[H]-5-HT uptake and lower basal release in both cortical and hippocampal slices (File *et al*, 1993). Reduced 5-HT availability (as shown by lower basal and K^+ evoked ^{3}[H]-5-HT release) has also been demonstrated in chicks following exposure to predator odour (Fluck *et al*, 1996).

6.1.3. Endocrinological changes following predator exposure

Laboratory animals demonstrate a variety of endocrinological changes in response to predator exposure. Groups of rats exposed to cat odour for the first time displayed significant increases in plasma corticosterone concentrations. However, this effect was found to habituate following five exposures (File *et al*, 1993) thus demonstrating a dissociation of behavioural and corticosterone responses to predator odour exposure.

Rat pups exposed to predator odour display significantly elevated corticosterone concentrations (Tanapat *et al*, 1998). Lu *et al* (1998) have also demonstrated elevated ACTH and plasma corticosterone levels in mice following exposure to rats. These findings may be influenced by the species of mouse used in the experiments as higher responses were observed in BALB/c than in C57 mice.

Increased ACTH and plasma corticosterone concentrations were reported in rats five minutes after cat exposure as compared to non-stressed controls (Adamec *et al*, 1998). This study also measured CRF, AVP and bombesin concentrations in specific regions of HPA circuitry. CRF levels were increased in the anterior hypothalamus and decreased in the dorsomedial hypothalamus. AVP concentrations were found to be reduced in the lateral hypothalamus. Bombesin is a neuropeptide which potently stimulates NA release from the adrenal medullae, ACTH from the pituitary and elicits behaviours typically associated with increased emotionality and arousal (Kent *et al*, 1998). Concentrations of this neuropeptide were altered in a number of hypothalamic areas following exposure of rats to cat odour (decreased in PVN and anterior hypothalamus / increased in ventromedial hypothalamic nuclei).

These changes indicate that the status of the HPA axis is significantly altered following predator (odour) exposure. These data also verify the predator exposure procedure as being a stressful stimulus to many animals as elevations of ACTH and the concomitant release of corticosterone are hallmarks of the stress response. Involvement of the HPA axis following predator exposure implies a helpless state (as described by Henry, 1993; see section 1.4.4) which may be associated with stress beyond control that could result in the manifestation of depressive symptoms.

Given the acute response of rats to predator exposure, it follows that applying this potential stressor in a chronic fashion may induce a long-term stress response in laboratory animals - this is what we have attempted to achieve in this chapter. Predator stress has not been applied in a chronic fashion in any previous studies, and the parameters being measured in the following studies have not yet been investigated. We decided to use BALB/c mice in our studies, as these are known to display high reactivity to stress (Lu *et al*, 1998; Karen Mellowdew, Institute of Psychiatry, personal communication) - with Brown Norwegian rats as the predator species (as these are not commonly kept in our animal facility and would possess a novel odour). Assessments of normal and stressed behaviours were made by measuring locomotor activity and anhedonic responses in the mice before and after acute and chronic exposure to the predator stressor. It was decided to use these measures for examining stress-related effects as these represent core symptoms of depressive syndromes and changes in these behaviours have been demonstrated numerous times following chronic stress in laboratory animals (Willner *et al*, 1987a; Monleon *et al*, 1995).

In addition to any behavioural changes, plasma corticosterone concentrations were assessed following acute and chronic exposure to the predator. Specific binding of ^{3}H-dexamethasone to cortical and hippocampal CR was also investigated following predator exposure and we were particularly interested to see if any reductions in brain CR would occur following chronic predator stress which would be consistent with a CR down-regulation hypothesis in depression.

6.2. ACUTE STRESS STUDY

These studies aimed to investigate the effects of acute exposure of BALB/c mice to a non-contact predator stressor in order to determine whether predator exposure was producing any stress in the mice. Parameters being measured in this study were open field activity, sucrose intake, plasma corticosterone and specific ^{3}H-dexamethasone binding to CR in the cortex and hippocampus.

6.2.1 Study design

Experiment 1 - The aim of the first investigation was to determine the immediate effects of the predator stress on open field activity, sucrose intake, plasma corticosterone concentrations and CR binding. In this experiment, mice were killed or tested for behavioural changes (in a separate room) immediately following exposure to the predator stress.

Experiment 2 aimed to determine residual effects of the initial predator exposure on the various parameters measured in experiment 1 and also any effects of predator odour over 24 hours. In this experiment, mice remained in the stress room for a 24 hour period following the initial exposure to predator stress (as described above) before measurements were recorded for the various parameters.

6.2.2. Methods

6.2.2.i. Animals

Male adult BALB/c mice used in these investigations were obtained and maintained as described in section 5.2. Mice were individually housed under a normal light:dark cycle (lights on 06.00-18.00hrs) with free access to standard laboratory food and water except when undergoing deprivation periods prior to sucrose testing. Brown Norwegian

rats obtained from the Institute of Psychiatry animal facilities were used as the predator stress stimulus. Rats were housed in pairs, in a separate room from the mice and were maintained on 12:12 hour light:dark (lights on 06.00-18.00 hours) cycles with free access to standard laboratory rat food and water.

6.2.2.ii. Predator stress procedure

All mice allocated to the stress groups were housed in the same room as the Brown Norwegian rats in order to be subjected continuously to a predator odour stress stimulus. This formed the constant long-term exposure to predator odour. All visual predator exposures, constituting acute visual contact with the predator, took place in the same room. All nesting material and food and water were removed from the mouse cage for the duration of the acute predator exposure thereby removing all objects under which the mice could possibly find shelter. Mouse cages were wedged into a large "playpen" filled with polystyrene foam pieces to stabilise the mouse cages. Three Brown Norwegian rats were released into the playpen for a 5 minute period and allowed to freely roam over the tops of the mouse cages, through which both mice and rats could clearly see each other but could not make physical contact.

6.2.2.iii. Open field tests

Open field tests were conducted in a separate room over a 5 minute duration in the morning during the light phase of the mice (as discussed in section 5.7.). Control animals were tested before the stress group to prevent any residual predator odour from stressed mice affecting the test. Data was analysed using Student t-tests or Mann-Whitney tests between the control and stress group (using GB-STAT v6.5).

6.2.2.iv. Sucrose intake tests

Sucrose intake tests were conducted using one bottle containing a 3% sucrose solution (determined from the sucrose gradient – see sections 5.4. and 5.7.). This was presented to the mice following a 3-hour food and water deprivation period for a three-hour test period (see sections 5.5 and 5.7). Prior to the introduction of the stressor, mice were trained to drink 3% sucrose over a period of 24 hours followed by four 3-hour sucrose consumption tests over two weeks. These tests allowed for the measurement of baseline values and also for the division of animals into sub-groups depending on their intake of sucrose. For experiment 1 of the acute stress studies, mice were exposed to the predator stress towards the end of the 3-hour deprivation period in order for sucrose intake to be measured immediately after exposure. Data were analysed using Students t-test to compare sucrose intakes in control and stress groups (GB-STAT v6.5).

6.2.2.v. Radioimmunoassay of plasma corticosterone concentrations

a) Preparation of plasma serum from mouse blood - Serum samples were prepared from trunk blood that was obtained from the sacrificed animals by decapitation and exsanguination using a funnel and collection tube. Approximately 2mL of blood were collected from each mouse. Following the removal of the blood from the animal, the samples were allowed to stand for approximately half an hour, then shaken to dislodge the clot. After 1-hour storage at 4°C, the samples were left at room temperature for 20 minutes prior to removal of the clot from the tube. The supernatant was transferred to a clean tube and spun at 3800 rpm for 10 minutes using a bench top centrifuge.

b) Determination of plasma corticosterone - Plasma corticosterone concentrations were quantified using a Gamma-B ^{125}I-corticosterone double antibody radioimmunoassay (RIA) kit (Immunodiagnostic Systems Ltd, Tyne & Wear, U.K.). Tubes were prepared in duplicate for total counts (TC), non-specific binding (NSB), maximum binding (Bo), corticosterone standards and samples as described in the RIA kit protocol. The amount of radioactivity in all tubes was counted for at least 1 minute in a gamma counter.

c) Calculation of results - Average counts (cpm) were determined for each set of duplicate assay tubes. Average NSB counts were subtracted from average Bo, standards and unknown samples and this number multiplied by 100 to give the % B / Bo for each sample.

$$\%\ B / Bo = \frac{\text{cpm (standard or unknown)} - \text{cpm (NSB)}}{\text{cpm (Bo)} - \text{cpm (NSB)}} \times 100$$

% B / Bo was plotted for each standard vs. standard concentration enabling the concentration of corticosterone in unknown samples to be read directly from this standard curve. Statistically significant differences between groups were determined using Students t-test to compare control and stress groups (GB-STAT v6.5).

6.2.2.vi. Specific binding of ^{3}H-dexamethasone to mouse cytosolic CR

All sacrifice and dissection procedures were conducted as described in section 2.3. Preparation of mouse cytosolic fractions was carried out essentially as described in section 2.3 (with the modifications made in section 5.6. in order to yield 3.3mg wet weight original tissue). Tissues were homogenised in 1:30 w/v assay buffer and, following centrifugation, 100µL aliquots were incubated with either 10% ethanol or 5µM hydrocortisone. ^{3}H-dexamethasone was added to the assay at concentrations ranging from 0.625 – 20nM for cortex samples and at a single concentration of 10nM

for hippocampus samples. Subsequent procedures and analysis was carried out as described in section 2.3. Statistical comparisons between the control and stress groups were made using Students t-test (GB-STAT v6.5).

6.2.3. Results

6.2.3.i. Open field tests

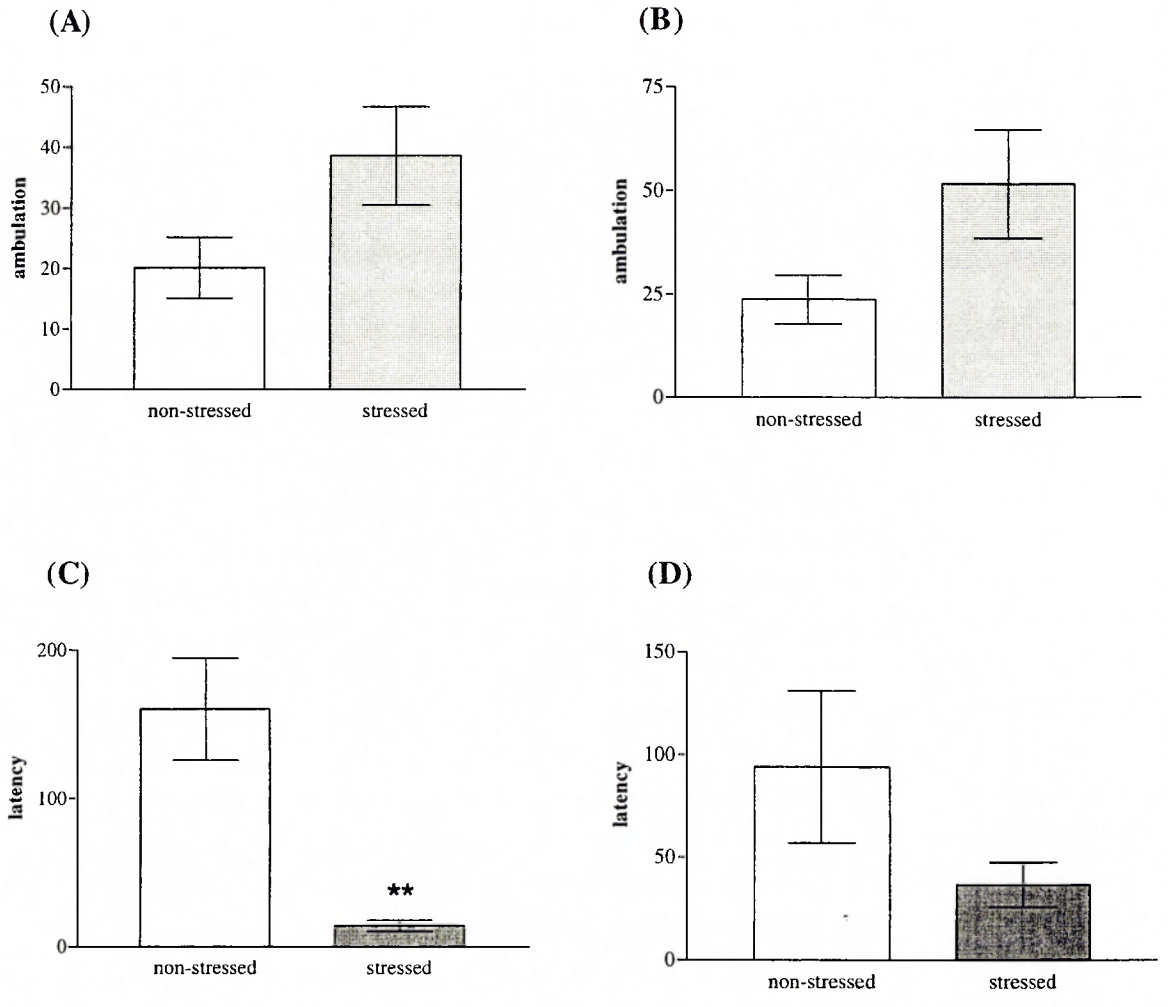
In both experiments 1 and 2, the exposure of BALB/c mice to predator stress increased ambulation scores in the open-field arena (Figures 6.2.3.i.A and B) however, these results did not reach statistical significance. There were no differences in grooming scores in both experiments 1 or 2. Rearing scores also did not differ between non-stressed and stressed groups in either experiment 1 or 2. Defecation was not significantly different in the stressed group immediately or 24 hours following predator stress as compared to non-stressed values. Animals subjected to the open field test immediately following predator stress displayed a significant reduction in mobility latency as compared to non-stressed controls (see Figures 6.2.3.i.C. and 6.2.3.i.D.). The latency to movement of stressed mice (37 seconds) in the open field 24 hours after predator stress was also lower than in control animals (94 seconds), however this effect was not significantly different from non-stressed values. See Appendix 6.2.3.A for OFT values for ambulation, grooming, rearing, defecation and mobility latency.

6.2.3.ii. Sucrose intake tests

Predator stress significantly reduced the sucrose intake of BALB/c mice as compared to non-stressed controls when tested immediately after exposure (Figure 6.2.3.ii.A.). Sucrose intake of stressed mice was not different from that of unstressed animals when tested 24 hours after exposure to predator stress (Figure 6.2.3.ii.B). See Appendix 6.2.3.B for sucrose intake values.

Figure 6.2.3.i.

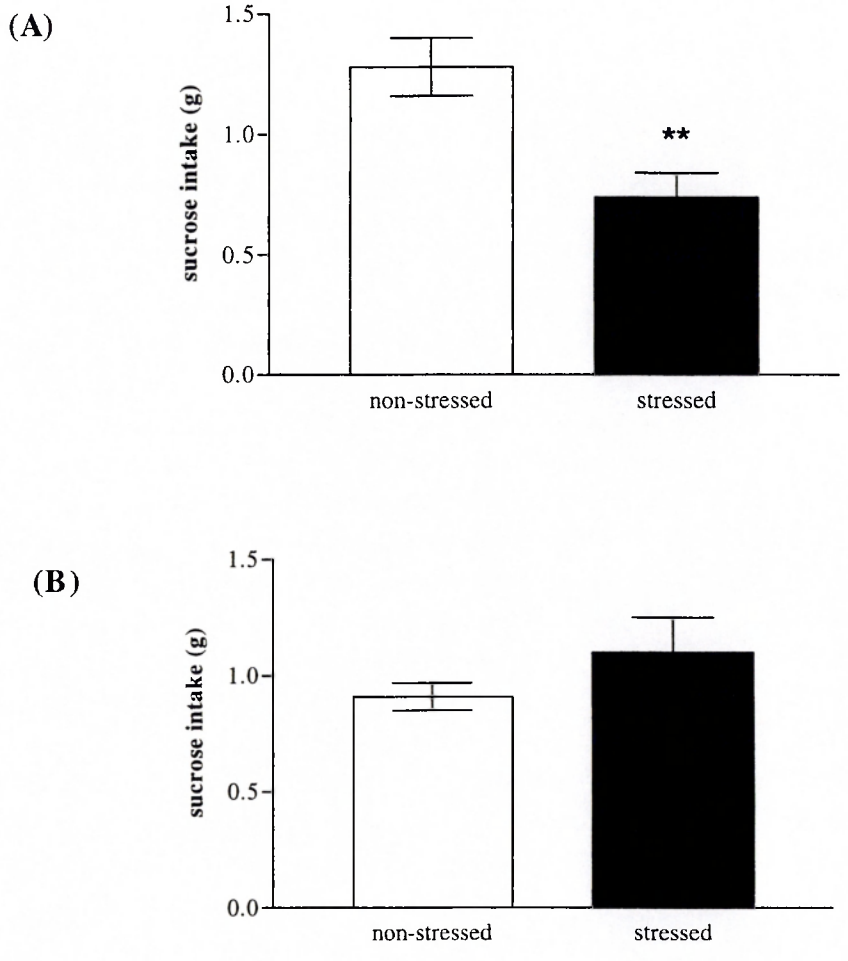
Open field activity of BALB/c mice following acute exposure to predator stress



BALB/c mice (n=8 per group) were subjected to open field tests immediately, or 24 hours after exposure to predator stress. Data are expressed as mean ± sem, see Appendix 6.1.A. (**A**) ambulation scores immediately following exposure to predator stress, (**B**) ambulation scores 24 hours after exposure to predator stress, (**C**) mobility latency immediately following exposure to predator stress, (**D**) mobility latency 24 hours after exposure to predator stress. Statistically significant differences were determined using t-tests (GB-STAT v6.5) and are denoted by ** ($p<0.01$).

Figure 6.2.3.ii.

Sucrose intake of BALB/c mice following acute exposure to predator stress



BALB/c mice (n=10 per group) were subjected to one-bottle sucrose consumption tests immediately or 24 hours after exposure to predator stress. Sucrose intake tests were conducted over a 3-hour period following 3-hours of food and water deprivation. Data are expressed as mean (± sem) intake in g, see Appendix 6.1. B. **(A)** sucrose intake immediately following exposure to predator stress, **(B)** sucrose intake 24 hours after exposure to predator stress. Statistically significant differences were determined using t-tests (GB-STAT v6.5) and are denoted by ** ($p<0.01$).

6.2.3.iii. Radioimmunoassay of plasma corticosterone

There were no statistically significant differences in plasma corticosterone concentrations between non-stressed and stressed animals immediately (Figure 6.2.3.iii.A) or 24 hours (Figure 6.2.3.iii.B) after predator exposure. See Appendix 6.2.3.C for plasma corticosterone values.

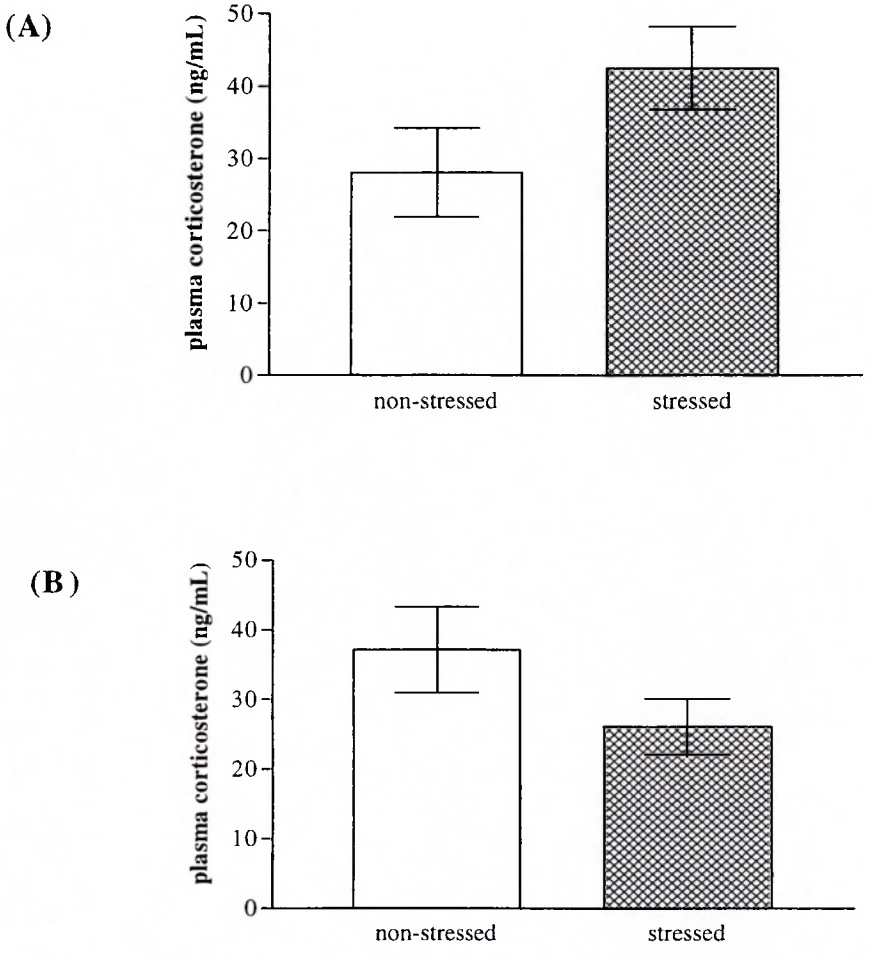
6.2.3.iv. ^{3}H-dexamethasone binding to cortical/hippocampal CR

There were no significant differences in the B_{max} or K_D of ^{3}H-dexamethasone binding to CR in cortex taken from animals killed immediately after predator stress compared to non-stressed values (Figures 6.2.3.iv.A. and B.). Values for B_{max} and K_D in cortex taken from mice that had been killed 24 hours after stress were also not significantly different from non-stressed values (see Figures 6.2.3.iv.C. and D.).

No significant differences were found between B_{max} values in hippocampus taken from animals sacrificed immediately or 24 hours after stress compared to non-stressed animals (Figures 6.2.3.iv.E. and F.). See Appendix 6.2.3.D for ^{3}H-dexamethasone binding values.

Figure 6.2.3.iii.

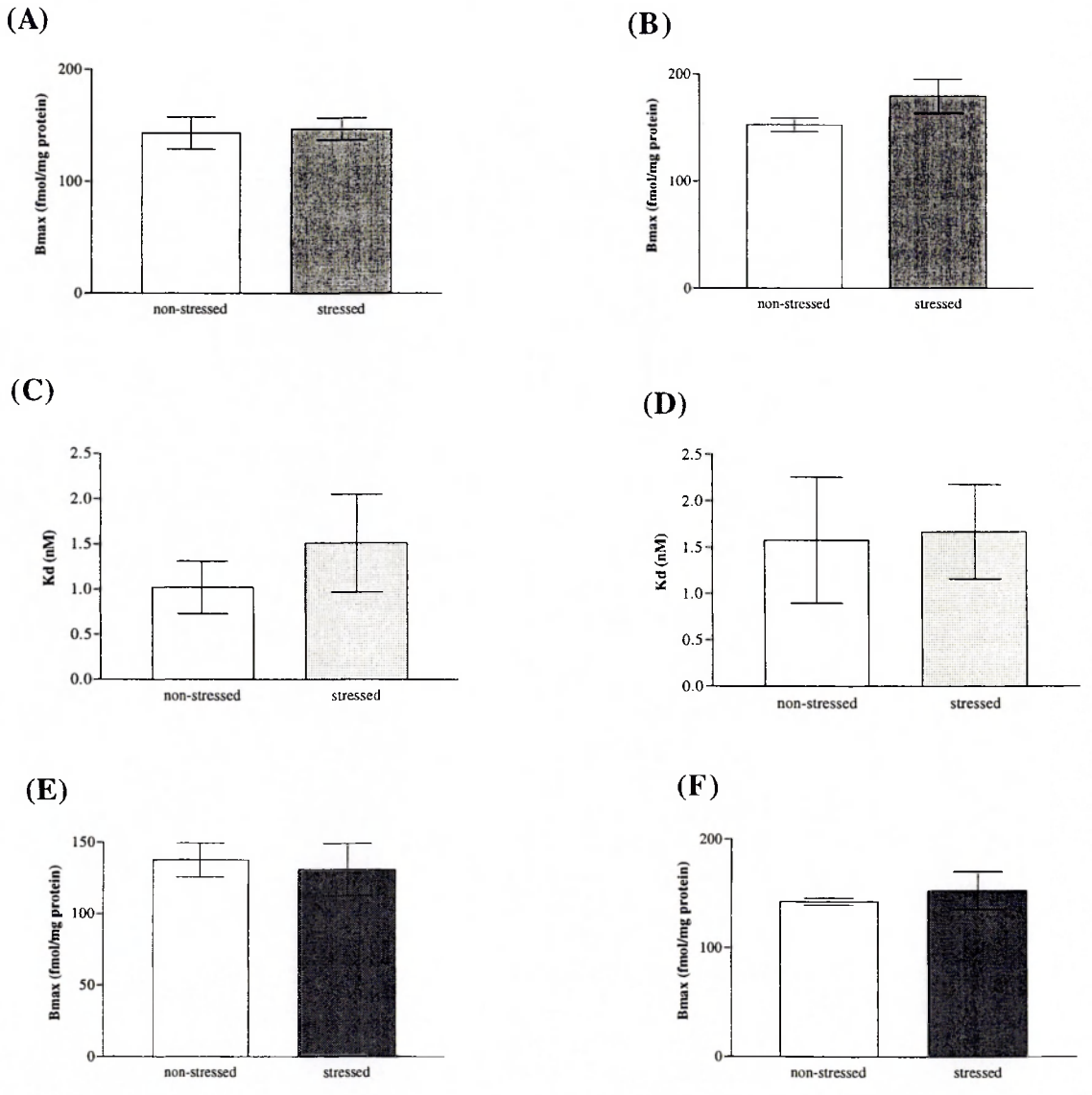
Plasma corticosterone concentrations in BALB/c mice following acute exposure to predator stress



Plasma corticosterone levels in BALB/c mice (n=6-7 per group) were assessed in samples taken immediately or 24 hours following exposure to predator stress. Concentrations of plasma corticosterone were quantified using the radioimmunoassay methods described in section 6.2.3.iii. Data are expressed as mean (±sem), see Appendix 6.1.C. **(A)** plasma corticosterone concentrations immediately following exposure to predator stress, **(B)** plasma corticosterone concentrations 24 hours after exposure to predator stress. No statistically significant differences were determined between control and stress groups.

Figure 6.2.3.iv.

Specific binding of ^{3}H-dexamethasone to cortical and hippocampal cytosolic fractions following acute exposure to predator stress



Specific ^{3}H-dexamethasone binding to CR in cortex/hippocampus (taken from BALB/c mice (n=7-8 per group)) was measured following acute exposure to predator stress. Samples were taken immediately or 24 hours after exposure to predator stress and cortical cytosolic fractions were prepared as described in sections 2.3 and 6.2.3.v. Data are expressed as mean ± sem, see Appendix 6.1.D. **(A)** B_{max} of cortical CR binding immediately following exposure to predator stress, **(B)** B_{max} of cortical CR binding 24 hours after exposure to predator stress, **(C)** K_D of cortical CR binding immediately following exposure to predator stress, **(D)** K_D of cortical CR binding 24 hours after exposure to predator stress, **(E)** B_{max} of hippocampal CR binding 24 hours after exposure to predator stress, **(F)** B_{max} of hippocampal CR binding 24 hours after exposure to predator stress . No statistically significant differences were found between control and stress groups.

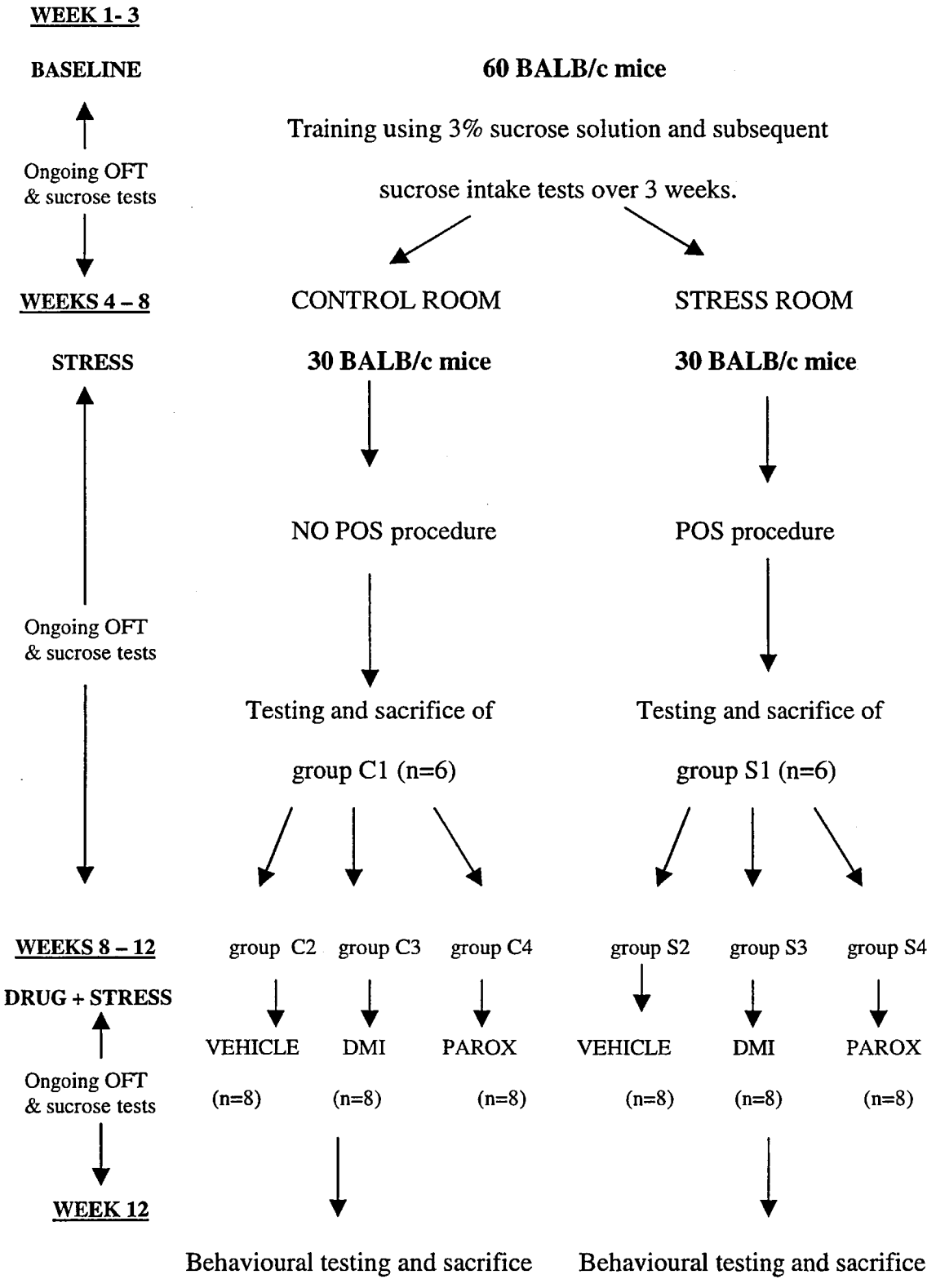
6.3. CHRONIC STRESS AND ANTIDEPRESSANT STUDIES

This study was designed to firstly, investigate the effects of chronic exposure to predator stress on various behavioural and neuroendocrine parameters measured in BALB/c mice and secondly, to ascertain the effects, if any, of long-term administration of DMI and paroxetine on these parameters.

6.3.1. Chronic stress and drug study design

60 BALB/c mice were initially exposed to bottles containing a 3% sucrose solution for a period of 48 hours, followed by a number of sucrose intake tests in order to train the animals to drink the sucrose solution and to establish baseline sucrose consumption values (Monleon *et al*, 1995). On the basis of these values, mice were divided into 8 groups (n=6-8) that were to undergo various control/stress and drug administration procedures (Figure 6.3.1).

Figure 6.3.1. Chronic predator stress and antidepressant drug study schedule



6.3.2. Methods

6.3.2.i. Animals

As described in section 6.2.2.i. Mice were weighed once weekly for the duration of the investigation.

6.3.2.ii. Predator stress procedure

All mice allocated to the stress group following initial sucrose intake tests were housed in a separate room, which was occupied by Brown Norwegian rats. This provided a constant, chronic exposure to predator odour, which was not considered to be stressful enough to produce behavioural changes in the mice (personal communication; Karen Mellowdew, Institute of Psychiatry, London.). Therefore, in addition to the continuous predator odour, an acute, visual predator stress was administered for different durations on different days at varying times and sometimes more than once daily. The duration and time of predator stress were varied in order to make the stressor more unpredictable for the mice and to minimise the possibility of habituation. Also, different Brown Norwegian rats were used as the stress stimulus in the presence/absence of food/water/nesting material (so the mice had nothing under which to shelter). The procedure described in section 6.2.2.ii. was repeated with all cages, until all the mice in the stress group had received acute exposure to the predator stress. A detailed stress schedule was kept throughout the study. Mice were subjected to predator stress for the first 5 weeks of the investigation (group S1) and also throughout the period of drug administration (groups S2, S3 and S4).

6.3.2.iii. Antidepressant drug administration

The antidepressant drugs used in this study were;

1) The tricyclic antidepressant, desmethylimipramine (DMI)

2) The selective serotonin reuptake inhibitor, paroxetine

Animals were randomly assigned to 6 groups comprised of 3 control groups and 3 stress groups. Each of the non-stress and stress groups were treated once daily p.o. (between 08.00 – 09.00 hours) with either distilled water vehicle, DMI (15mg/kg) or paroxetine (7.5 mg/kg). Animals were dosed in this manner for 28 days during which, the stress groups were also undergoing exposure to predator stress. Drug/vehicle administration began following a 4 week exposure to predator stress.

6.3.2.iv. Body weights

Body weights of all BALB/c mice in the study were recorded once weekly. Data were analysed using analysis of variance followed by Student's t-tests (GB-STAT).

6.3.2.v.Open field tests

Open field-testing was conducted as described in sections 5.3. and 5.7, and was conducted over two days for ease of operation and in order to keep test times between 08.00 – 11.00 hours on each day. Equal numbers of mice from each group were tested on each day to minimise bias between groups. Tests were carried out with the BALB/c mice every two weeks over the 14-week investigation in an attempt to establish normal activity and activity following predator stress and antidepressant administration. Results were analysed using analysis of variance or non-parametric tests followed by Student's t-test or Mann Whitney U-tests (GB-STAT v6.5).

6.3.2.vi. Sucrose intake tests

The intake of a 3% sucrose solution by the mice was used as a measure of hedonic responsiveness in these studies. One-bottle consumption tests were conducted following a 3-hour food and water deprivation period (see sections 5.4, 5.5 and 5.7). Sucrose intake tests were conducted twice a week over the 14-week investigation. Results were analysed using analysis of variance followed by Student's t-test (GB-STAT v6.5). Water intake was also monitored over three 3-hour test periods in the baseline, stress and drug administration periods of the investigation.

6.3.2.vii. Radioimmunoassay of plasma corticosterone

The radioimmunoassay of plasma corticosterone samples was conducted as described in section 6.2.2.v. Samples were randomised in order to minimise bias on each assay day. Results were analysed using analysis of variance followed by Student's t-tests (GB-STAT v6.5).

6.3.2.viii. Specific ^{3}H-dexamethasone binding to mouse cytosolic CR

CR binding assays were conducted in cortical and hippocampal tissues as described in section 6.2.2.vi.. Samples from each group were randomised to minimise bias on each experimental day. Results were analysed using analysis of variance followed by Student's t-tests using GB-STAT v6.5.

6.3.2.viiii. Calculations and analysis

Data were tested for statistical significance using ANOVA or non-parametric Kruskal-Wallis tests (GB-STAT v6.0) followed by Student's t-tests or Mann-Whitney tests where the F-ratio was significant ($p<0.05$).

6.3.3. Results

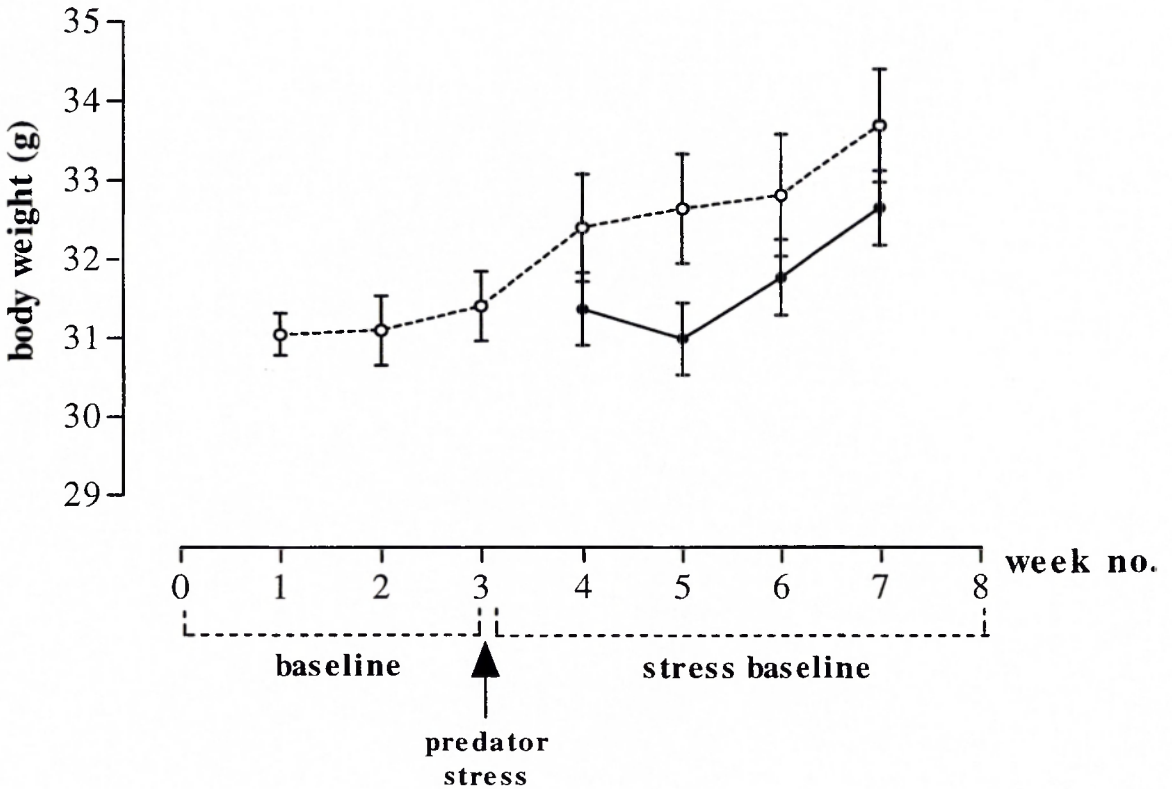
6.3.3.i. Body weights

Body weights of mice were consistently lower in the stressed animals than in non-stressed groups following introduction of the stress stimulus. This effect was not statistically significant; the maximum difference in weights between non-stressed and stressed groups was 5% at week 5 (Figure 6.3.3.i.(1). The stress factor separately had a significant effect on body weights. No effect of time and no significant ANOVA interactions of stress x time were observed.

In the vehicle treated groups, body weights of stressed animals were significantly lower than the non-stressed group at weeks 9, 10, 11 and 12 (Figure 6.3.3.i.(2)). In the DMI treated groups, there were no statistically significant differences between body weights of non-stressed and stressed animals but stressed animals had consistently lower body weights than non-stressed controls. Administration of paroxetine had no statistically significant effects on the body weights of non-stressed and stressed mice. A significant ANOVA interaction between drug treatment and time was observed on body weights following analysis of variance. See Appendix 6.3.3.A for body weight values.

Figure 6.3.3.i.(1)

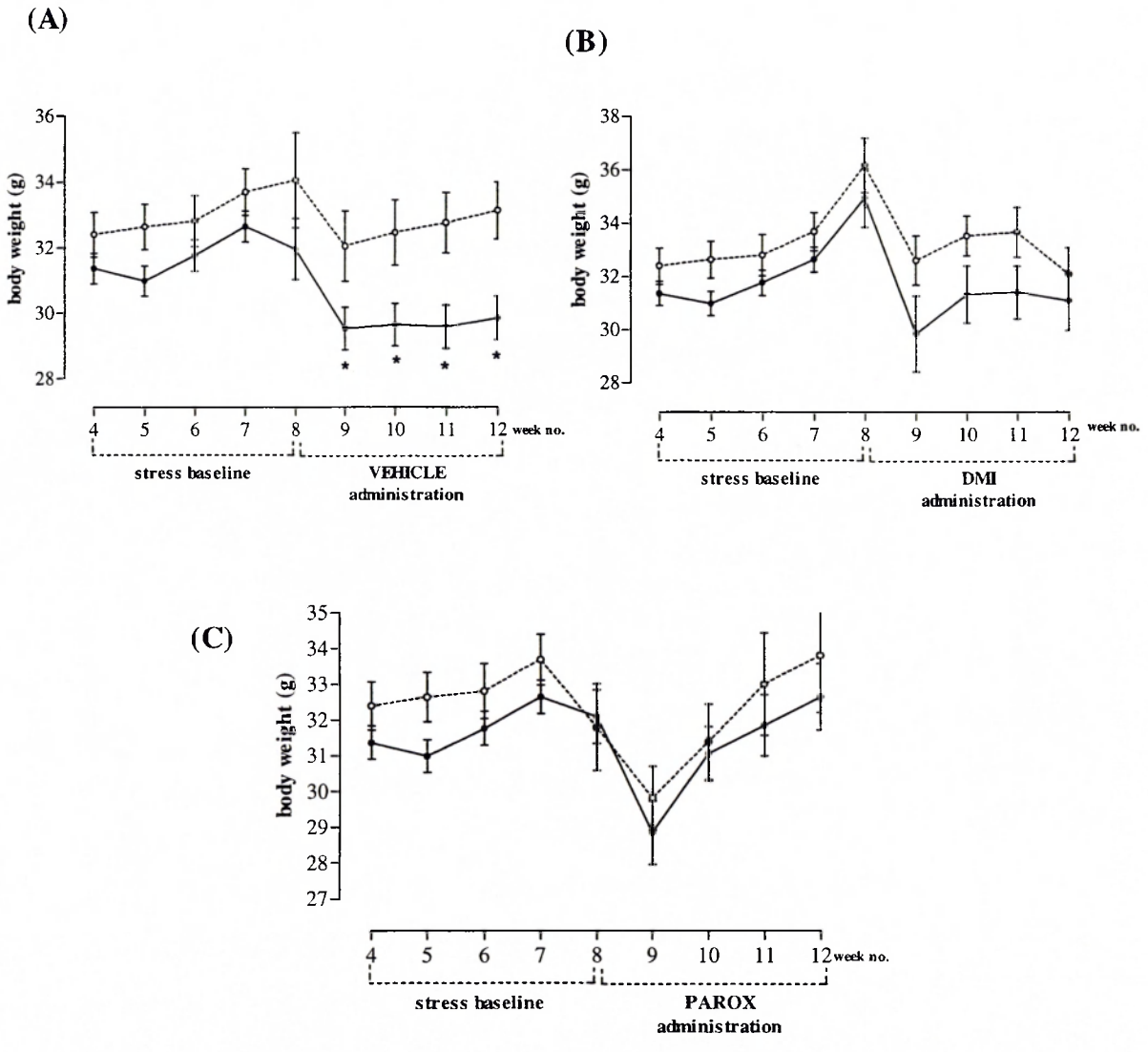
Effect of predator stress on body weights of BALB/c mice.



Body weights of individually housed BALB/c mice (n=7-8) were measured before and after the introduction of a predator stress stimulus (see section 6.3.2.iv). Data are expressed as mean ± sem, see Appendix 6.3.A. No statistically significant differences were observed using ANOVA followed by Students t-test (GB-STAT v6.5). ***Open circles = non-stressed group, closed circles = stressed group.*** ***ANOVA;*** *effect of stress – F (1, 239)= 7.84, p=0.006; effect of time- F (3, 239)= 2.14, p=0.095; interaction stress x time- F (3, 239)= 0.13, p= 0.94.*

Figure 6.3.3.i.(2)

Effects of chronic exposure to predator stress and administration of vehicle (A), DMI (B) and paroxetine (C) on the body weights of BALB/c mice



Body weights of BALB/c mice (n=7-8) were recorded over 8 weeks before and after the introduction of a predator stress stimulus (see section 6.3.2.iv). Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5mg/kg) commenced at week 8 for 28 days. Data are expressed as mean ± sem, see Appendix 6.3.A. Statistically significant differences were determined using ANOVA followed by Student's t-tests (GB-STAT v6.5) and are denoted by *p<0.05. ***Open circles = non-stressed group, closed circles = stressed group. ANOVA;*** *effect of stress – F (1, 206)= 22.7, p<0.0001; effect of treatment- F (2, 206)= 8.91, p= 0.022; effect of time- F (4, 206)= 6.97, p<0.0001; interaction stress x treatment F (2, 206)= 2.71, p= 0.069; interaction stress x time- F (4, 206)= 0.31, p= 0.87; interaction treatment x time- F (8, 206)= 2.15, p= 0.033; interaction stress x treatment x time- F (8, 206)= 0.13, p= 1.0.*

6.3.3.ii. Open field tests

Ambulation scores were significantly reduced in the stress group immediately following the introduction of the predator stress stimulus (Figure 6.3.3.ii.(1)). Two weeks after the introduction of predator stress, there were no significant differences in ambulation scores between stressed and non-stressed groups. Ambulation scores were also significantly lower in stressed animals following 4 weeks of vehicle administration (Figure 6.3.3.ii.(2).A). No statistically significant differences were observed in either stressed or non-stressed groups following the administration of DMI or paroxetine (Figures 6.3.3.ii.(2).B and C).

The time taken for the animal to start moving following its placement in the open-field arena (mobility latency) was significantly increased in mice subjected to predator stress for two and four weeks (Figure 6.3.3.ii.(3)). This effect was not observed after the beginning of vehicle administration or the administration of DMI or paroxetine. There were no significant differences between non-stressed and stressed groups following drug administration (Figure 6.3.3.ii.(4)). A significant effect of stress on mobility latency was revealed following ANOVA. No significant interactions between stress, drug treatment and time were observed for this parameter.

Defecation counts in the open-field were significantly reduced immediately after the introduction of predator stress but were no different to non-stressed values after 4 weeks of predator exposure (Figure 6.3.3.ii.(5)). Defecation counts were also significantly different in stressed animals following vehicle administration over 4 weeks as compared to the non-stressed controls (Figure 6.3.3.ii.(6)). There were no significant differences between stressed and non-stressed groups following the

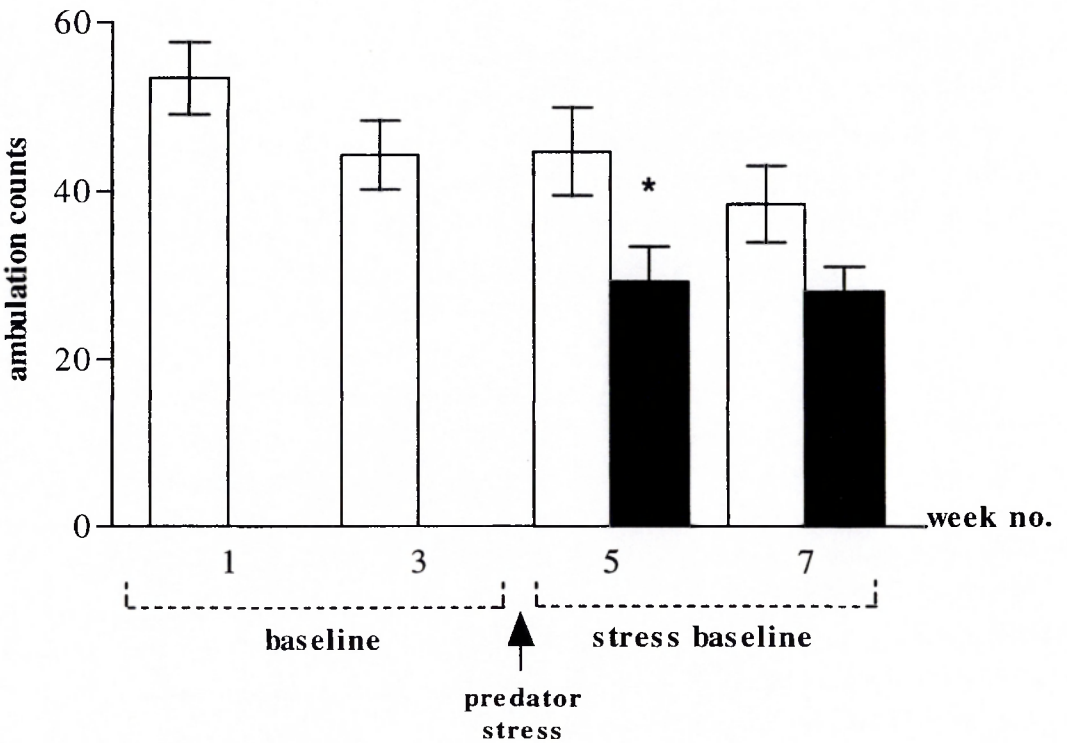
administration of DMI and paroxetine. Significant effects of stress and drug administration were revealed on defecation counts, with significant interactions between stress x drug administration also observed following ANOVA.

Grooming scores were found to be very erratic and no significant differences or interactions between any of the groups or factors investigated could be determined. Data is shown in Appendix 6.3.3.B.

The data obtained for rearing of mice in the open-field arena was also found to be irregular and no trends or significant differences or interactions between any of the groups or factors could be ascertained. Data is shown in Appendix 6.3.3.B.

Figure 6.3.3.ii.(1)

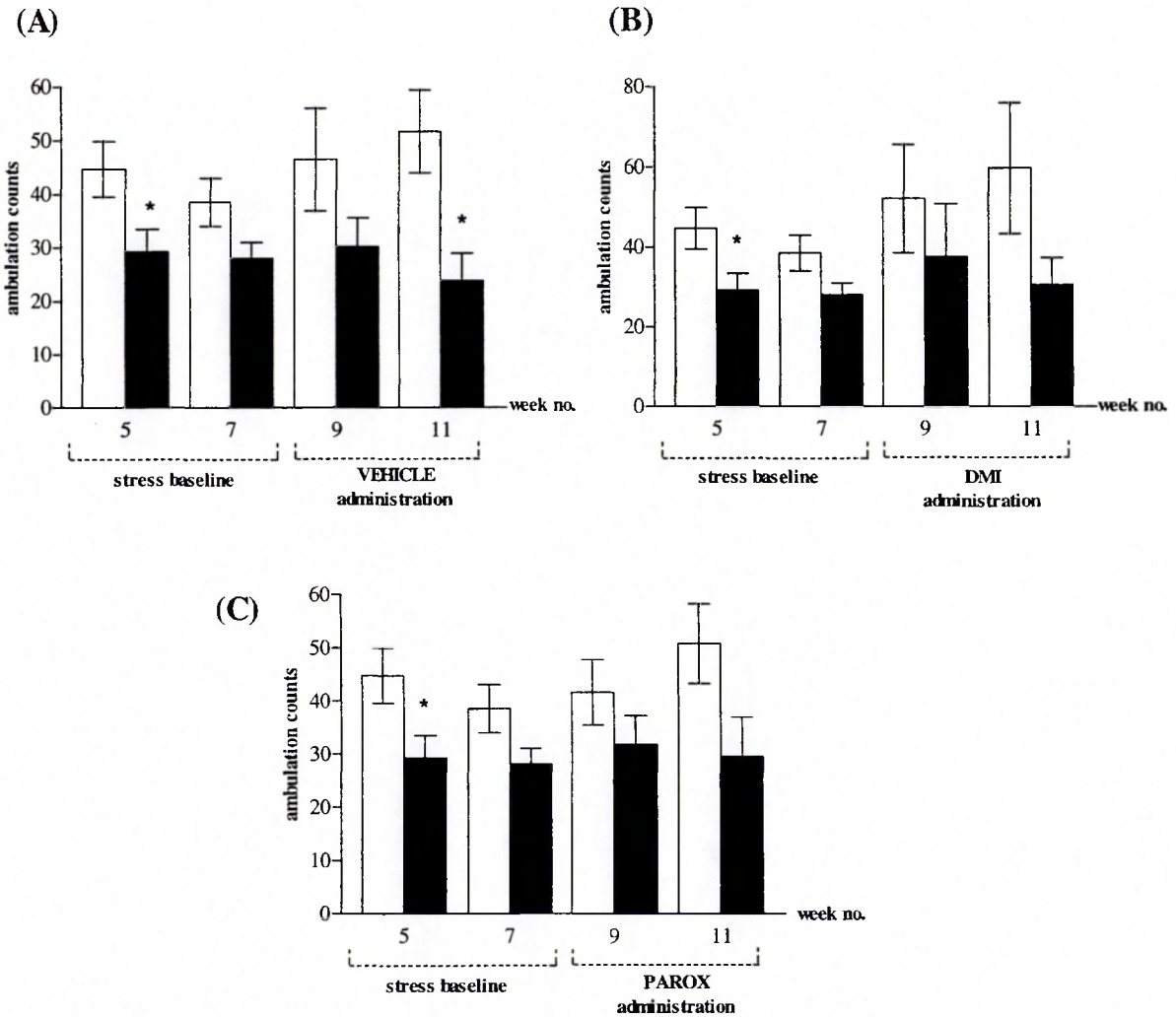
Effects of predator stress on ambulation scores of BALB/c mice



Ambulation counts of individually housed BALB/c mice were measured (in the open-field arena, see section 6.3.2v) before and after the introduction of a predator stress stimulus. Data were expressed as mean ± sem, see Appendix 6.3.B. Statistically significant differences were determined by ANOVA followed by Student's t-test (GB-STAT) and are denoted by *p<0.05. ***Open bars = non-stressed group, closed bars = stressed group. ANOVA;*** *effect of stress – F (1, 119)= 9.14, p=0.003; effect of time- F (1, 119)= 0.75, p=0.39; interaction stress x time- F (1, 119)= 0.34, p= 0.56.*

Figure 6.3.3.ii.(2)

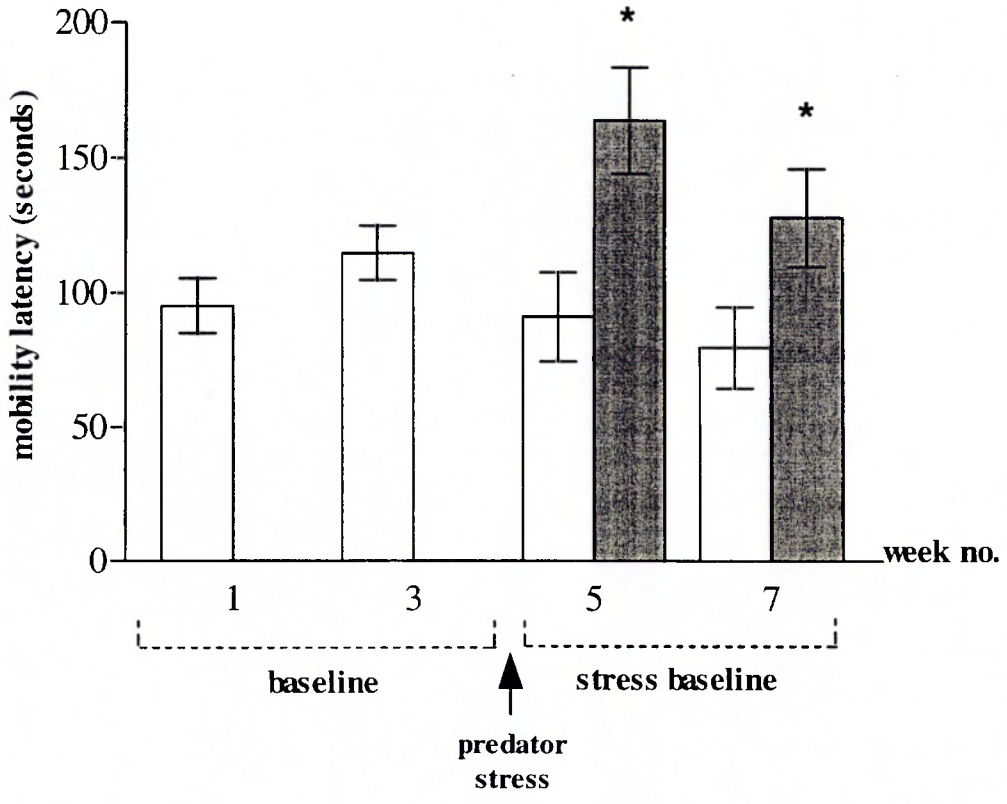
Effects of chronic exposure to predator stress and administration of vehicle (A), DMI (B) and paroxetine (C) on ambulation scores of BALB/c mice



Ambulation scores of BALB/c mice were measured, (using the open-field arena - see section.6.3.2.v) over 8 weeks (4 weeks before, and after the introduction of a predator stress stimulus). Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5mg/kg) began at the start of week 8 for 28 days. Data are expressed as mean ± sem, see Appendix 6.3.B. Statistically significant differences were determined using ANOVA followed by Student's t-tests (GB-STAT v6.5) and are denoted by *p<0.05. ***Open bars = non-stressed group, closed bars= stressed group. ANOVA;*** *effect of stress – F (1, 82)= 2.1, p=0.15; effect of treatment- F (2, 82)= 1.25, p= 0.29; effect of time- F (1, 82)= 0.21, p=0.65; interaction stress x treatment F (2, 82)= 0.13, p= 0.88; interaction stress x time- F (1, 82)= 15.89, p= 0.0001; interaction treatment x time- F (2, 82)= 0.08, p= 0.92; interaction stress x treatment x time- F (2, 82)= 0.34, p= 0.72.*

Figure 6.3.3.ii.(3)

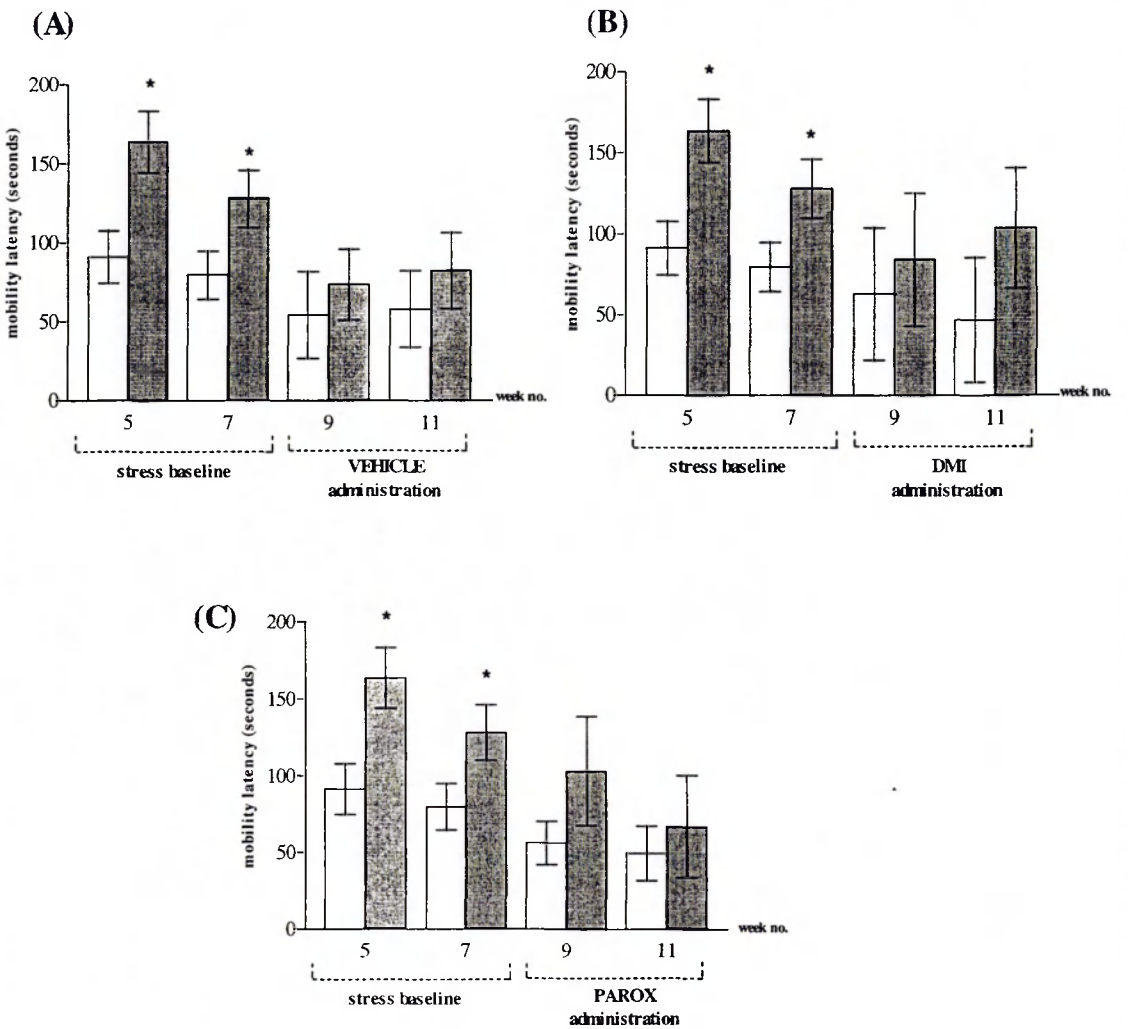
Effects of predator stress on the mobility latency of BALB/c mice.



The mobility latency (time after beginning of test at which movement started) of individually housed BALB/c mice was measured (in the open-field arena - see section 6.3.2.v) before and after the introduction of a predator stress stimulus. Data are expressed as mean ± sem, see Appendix 6.3.B. Statistically significant differences were determined by ANOVA followed by Student's t-test (GB-STAT v6.5) and are denoted by **p<0.05. ***Open bars = non-stressed group, closed bars = stressed group. ANOVA;*** *effect of stress – F (1, 119)= 11.97, p=0.0008; effect of time- F (1, 119)= 1.83, p=0.18; interaction stress x time- F (1, 119)= 0.49, p= 0.49.*

Figure 6.3.3.ii.(4)

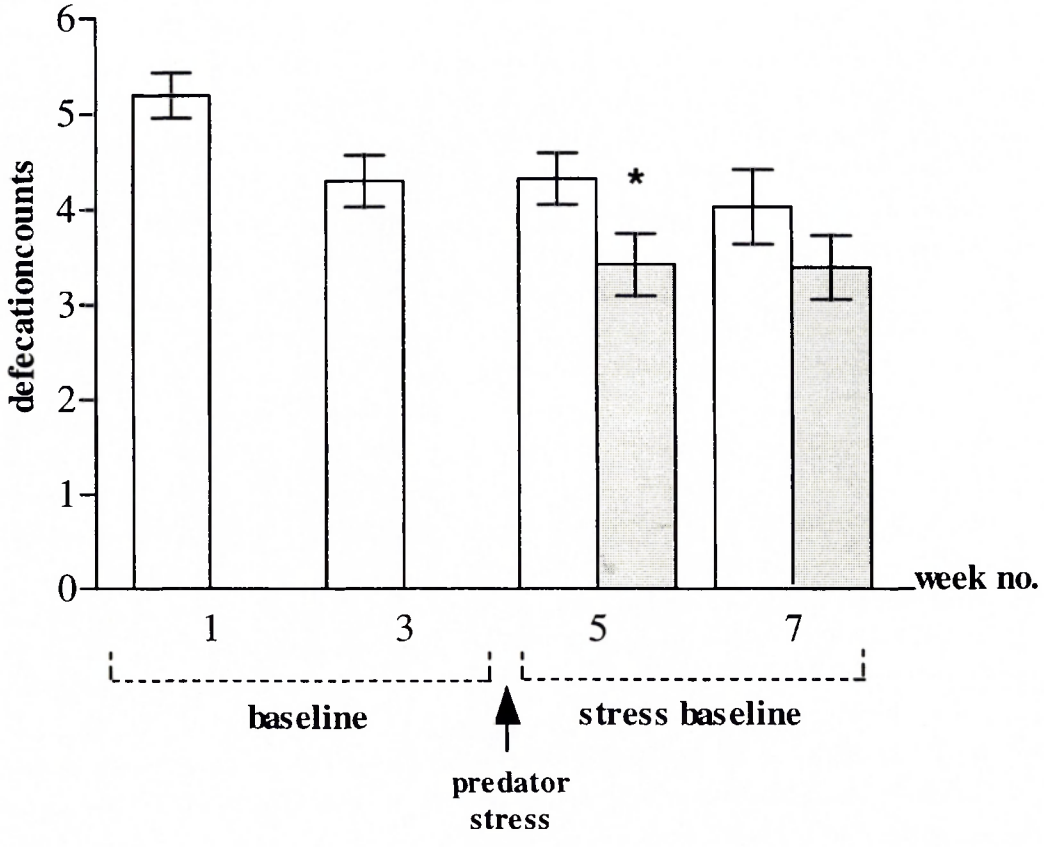
Effects of chronic exposure to predator stress and administration of vehicle (A), DMI (B) and paroxetine (C) on the mobility latency of BALB/c mice



Mobility latency (time after beginning of test that movement was started) of BALB/c mice was measured (in the open-field arena - see section 6.3.2.v) over 8 weeks (4 weeks before, and after the introduction of a predator stress stimulus). Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5mg/kg) began at the start of week 8 for 28 days. Data are expressed as mean ± sem, see Appendix 6.3.B. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by *p<0.05. ***Open bars = non-stressed group, closed bars= stressed group. ANOVA;*** *effect of stress – F (1, 82)= 4.77, p=0.032; effect of treatment- F (2, 82)= 0.65, p= 0.52; effect of time- F (1, 82)= 0.48, p=0.49; interaction stress x treatment F (2, 82)= 0.47, p= 0.62; interaction stress x time- F (1, 82)= 0.21, p= 0.64; interaction treatment x time- F (2, 82)= 0.27, p= 0.76; interaction stress x treatment x time- F (2, 82)= 0.09, p= 0.91*

Figure 6.3.3.ii.(5)

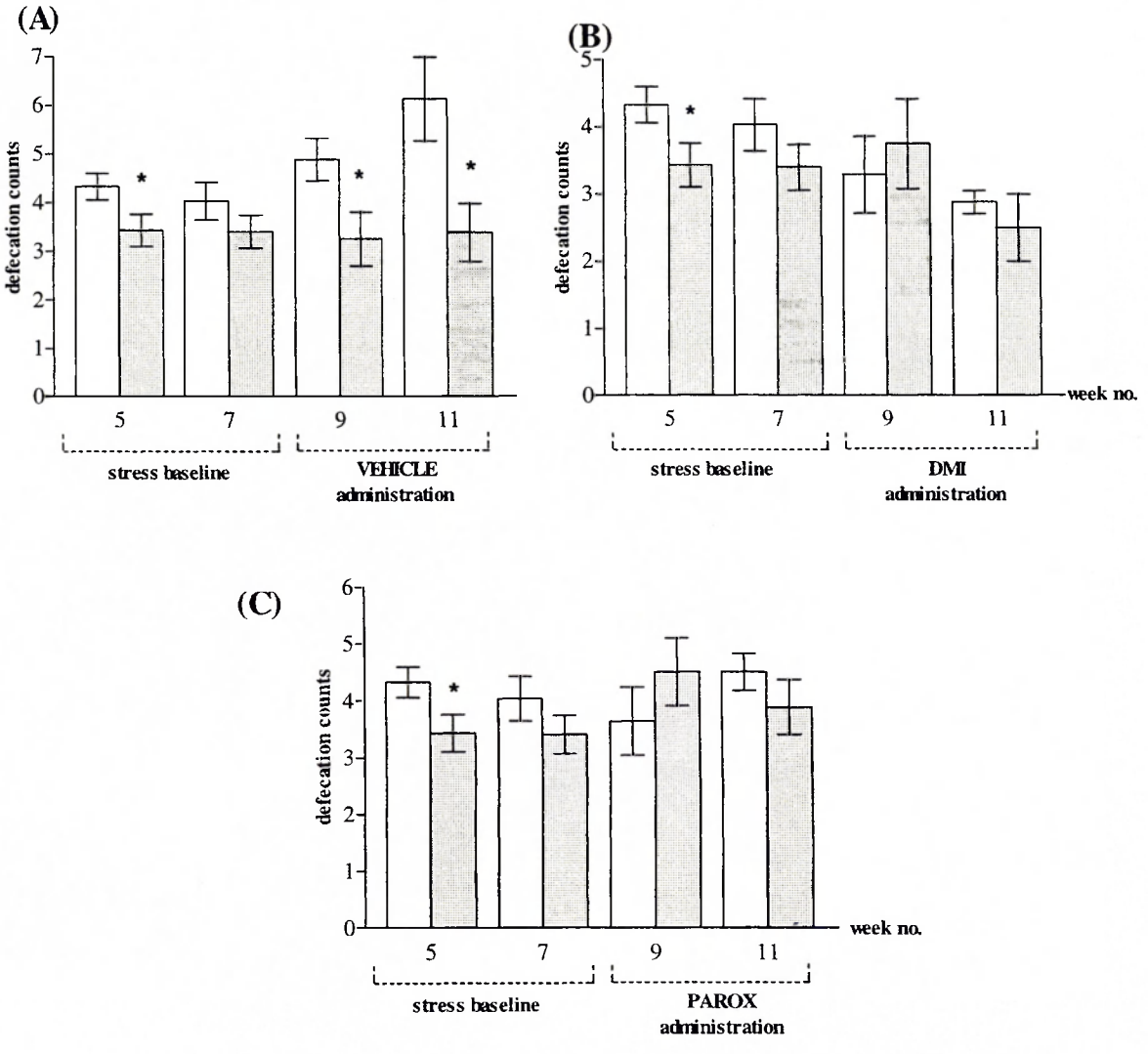
Effects of predator stress on defecation counts of BALB/c mice.



Defecation counts of individually housed BALB/c mice were measured (in the open-field arena - see section 6.3.2.v) before and after the introduction of a predator stress stimulus. Data were expressed as mean ± sem, see Appendix 6.3.B. Statistically significant differences were determined by ANOVA followed by Student's t-test and are denoted by *p<0.05. ***Open bars = non-stressed group, closed bars = stressed group. ANOVA;*** *effect of stress – F (1, 119)= 5.18, p=0.025; effect of time- F (1, 119)= 0.25, p=0.62; interaction stress x time- F (1, 119)= 0.16, p= 0.69.*

Figure 6.3.3.ii.(6)

Effects of chronic exposure to predator stress and administration of vehicle (A), DMI (B) and paroxetine (C) on defecation counts of BALB/c mice



Defecation counts of BALB/c mice were measured, (in the open-field arena - see section 6.3.2.v) over 8 weeks (4 weeks before, and after the introduction of a predator stress stimulus). Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5mg/kg) commenced at the start of week 8 for 28 days. Data are expressed as mean ± sem, see Appendix 6.3.B. Statistically significant differences were determined using ANOVA followed by Student's t-test (GB-STAT v6.5) and are denoted by *p<0.05. *Open bars = non-stressed group, closed bars= stressed group.* ***ANOVA;*** *effect of stress – F (1, 82)= 5.17, p= 0.026; effect of treatment- F (2, 82)= 4.95, p= 0.009; effect of time- F (1, 82)= 0.04, p=0.85; interaction stress x treatment F (2, 82)= 4.99, p= 0.009; interaction stress x time- F (1, 82)= 3.93, p= 0.05; interaction treatment x time- F (2, 82)= 1.36, p= 0.26; interaction stress x treatment x time- F (2, 82)= 0.03, p= 0.97.*

6.3.3.iii. Sucrose intake tests

Sucrose intake values in predator exposed groups were significantly reduced at weeks 3, 4, 5, 6 and 7 following the introduction of the predator stress stimulus. These changes were somewhat erratic with no differences in sucrose intake between non-stressed and stressed animals in three of the nine consumption tests conducted over the stress baseline period (Figure 6.3.3.iii.(1)). Reductions in sucrose intake ranged from 2% - 49 % of non-stressed values over all of the tests. Significant effects of stress and time were observed following analysis of variance alongside a significant stress x time interaction.

During the subsequent period of vehicle administration, sucrose intakes in stressed animals were also significantly lower than those in the non-stressed animals in most of the consumption tests conducted over this period. (Table 6.3.3.iii. and Figure 6.3.3.iii.(2)); these effects were also somewhat erratic. The chronic administration of DMI brought sucrose intake values in non-stressed and stressed groups closer to each other with fewer statistically significant differences being observed between the groups (Figure 6.3.3.iii.(2).B). Chronic paroxetine administration stabilised sucrose intake values in both stressed and non-stressed groups and also bought these values closer together (Table 6.3.3.iii and Figure 6.3.3.iii.(2).C). At week 12 of drug administration, there were no significant differences in sucrose intakes between DMI / paroxetine administered stressed and non-stressed animals. Significant separate effects of stress and time on sucrose intake were revealed following ANOVA however no significant interactions between stress, drug treatment and time were observed. See Appendix 6.3.3.C for intake values.

Sucrose intake data, when expressed as mg sucrose intake/g body weight, also displayed a general decrease in sucrose intake in relation to reductions in body weights in predator exposed groups (Figures 6.3.3.iii.(3) and 6.3.3.iii(4)) and followed a similar pattern to that of sucrose intake in drug administered groups (Appendix 6.3.3.D). There were no differences in water intake in any the groups. Water intake remained constant throughout the investigation (Table 6.3.3.iii).

Significant effects of stress and time were revealed on sucrose intake following ANOVA however no significant interactions of stress, drug treatment or time were observed.

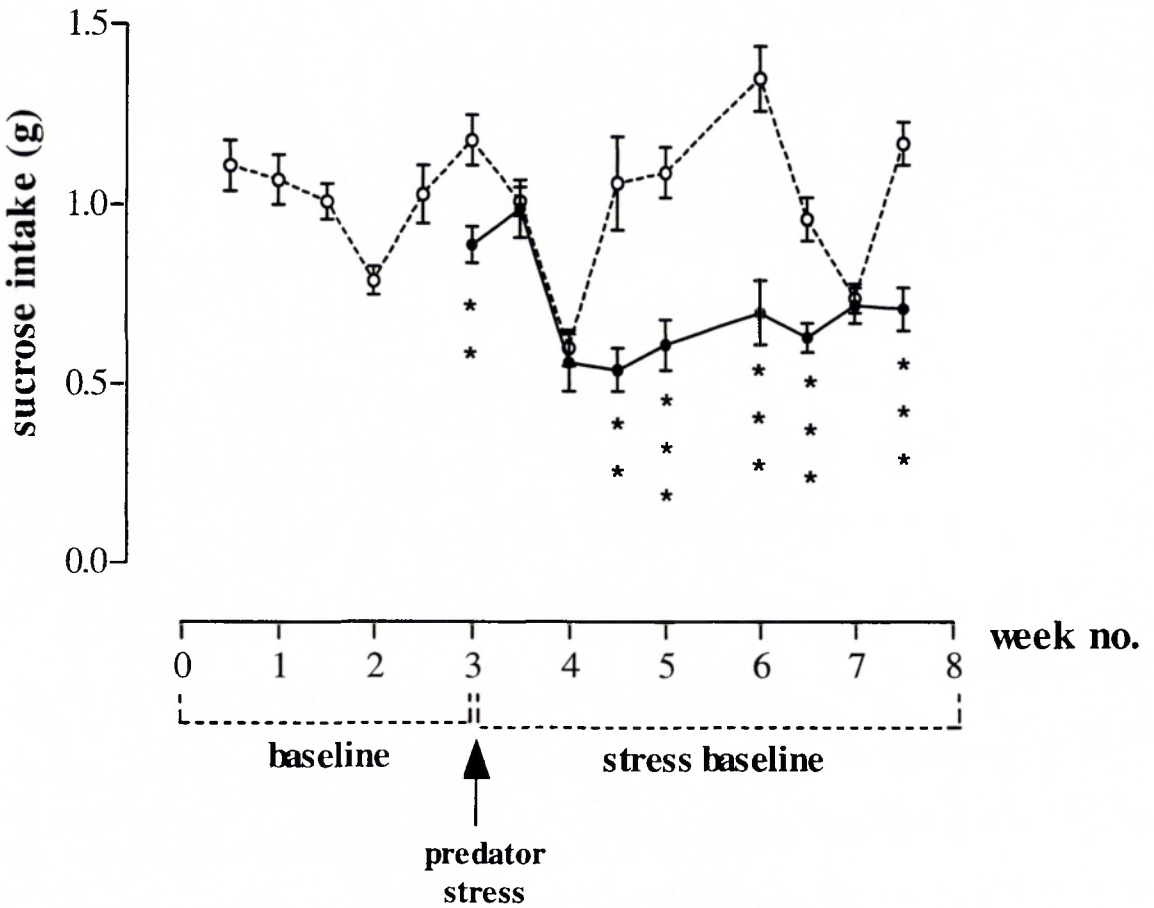
Table 6.3.3.iii Average water/sucrose intake values in BALB/c mice following baseline measures, introduction to predator stress and administration of vehicle/antidepressants.

	WATER non-stress	WATER stress	SUCROSE non-stress	SUCROSE stress
Baseline (week 1)	0.5 ± 0.03 (n=60)	---	1.11 ± 0.07 (n=60)	---
Stress baseline (week 6)	0.48 ± 0.04 (n=30)	0.52 ± 0.03 (n=30)	0.96 ± 0.06 (n=30)	0.63 ± 0.04 * (n=30)
Vehicle (week 10)	0.47 ± 0.07 (n=8)	0.47 ± 0.1 (n=8)	0.86 ± 0.15 (n=8)	0.56 ± 0.05 * (n=8)
DMI (week 10)	0.49 ± 0.08 (n=8)	0.5 ± 0.09 (n=8)	0.84 ± 0.12 (n=8)	0.92 ± 0.36 (n=8)
Paroxetine (week 10)	0.46 ± 0.07 (n=8)	0.44 ± 0.09 (n=8)	0.75 ± 0.08 (n=8)	0.36 ± 0.05 * (n=8)

Data are expressed as mean ± sem for week 1, 6 and 10 of the study. Statistically significant differences were determined using Student's t-tests or ANOVA and are denoted by *$p<0.05$ as compared to non-stressed controls. ***ANOVA;*** *effect of stress – $F(1, 47)= 0.004$, $p=0.96$; effect of time- $F(2, 47)= 0.14$, $p=0.87$; interaction stress x time- $F(2, 47)= 0.026$, $p= 0.97$.*

Figure 6.3.3.iii. (1)

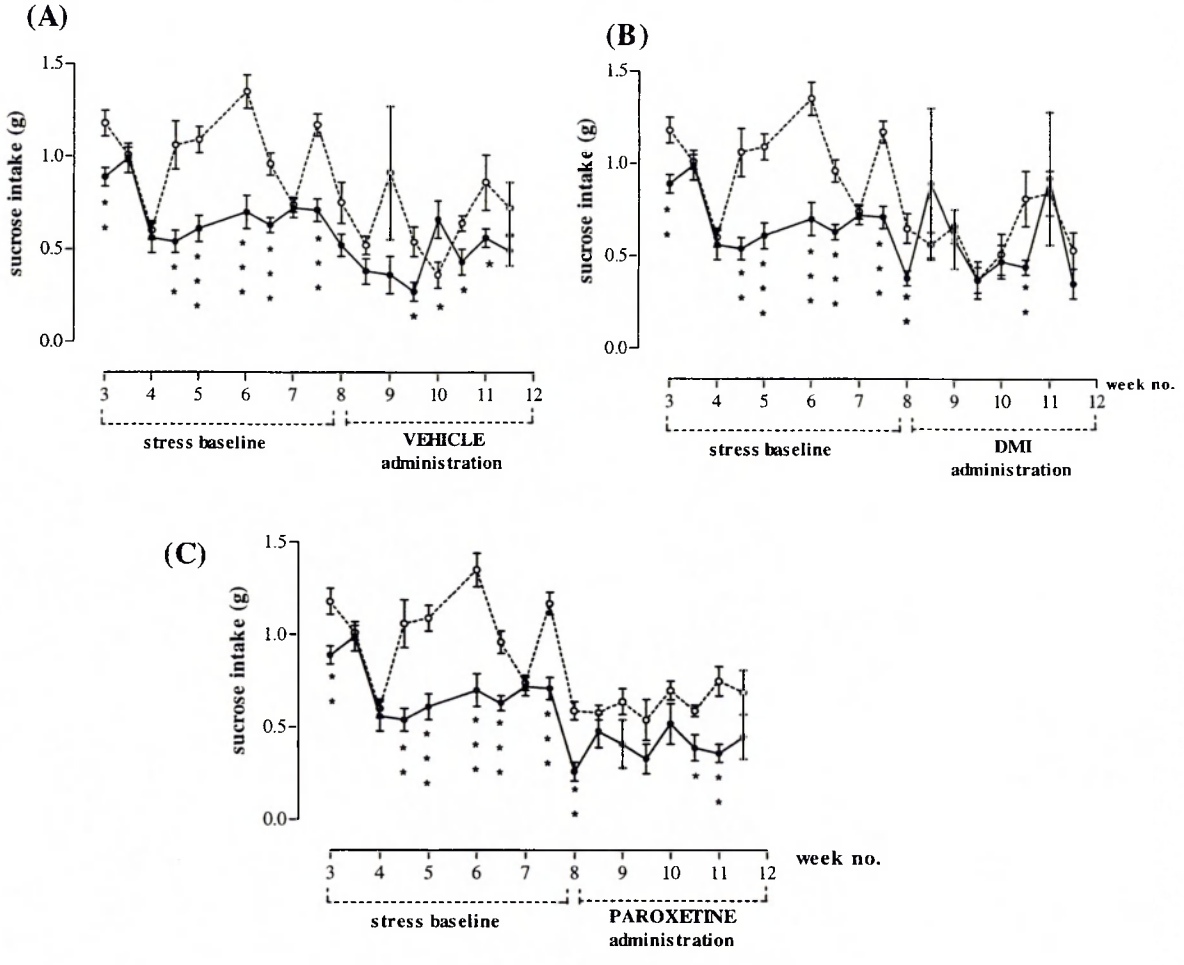
Effects of predator stress on the sucrose intake of individually housed BALB/c mice.



Intake of a 3% sucrose solution by BALB/c mice was measured using one-bottle consumption tests over a 3 hour period following a 3-hour food and water deprivation period (see section. 6.3.3.iii). Tests were conducted over 8 weeks (4 weeks before, and after the introduction of a predator stress stimulus). Data are expressed as mean ± sem, see Appendix 6.3.C. Statistically significant differences were determined using ANOVA followed by Student's t-test (GB-STAT v6.5) and are denoted by **p<0.01 and *** p<0.001. ***Open circles = non-stressed group, closed circles = stressed group. ANOVA;*** *effect of stress – F (1, 597)= 111.9, p<0.0001; effect of time- F (9, 597)= 30.08, p<0.0001; interaction stress x time- F (9, 597)= 8.77, p<0.0001.*

Figure 6.3.3.iii.(2)

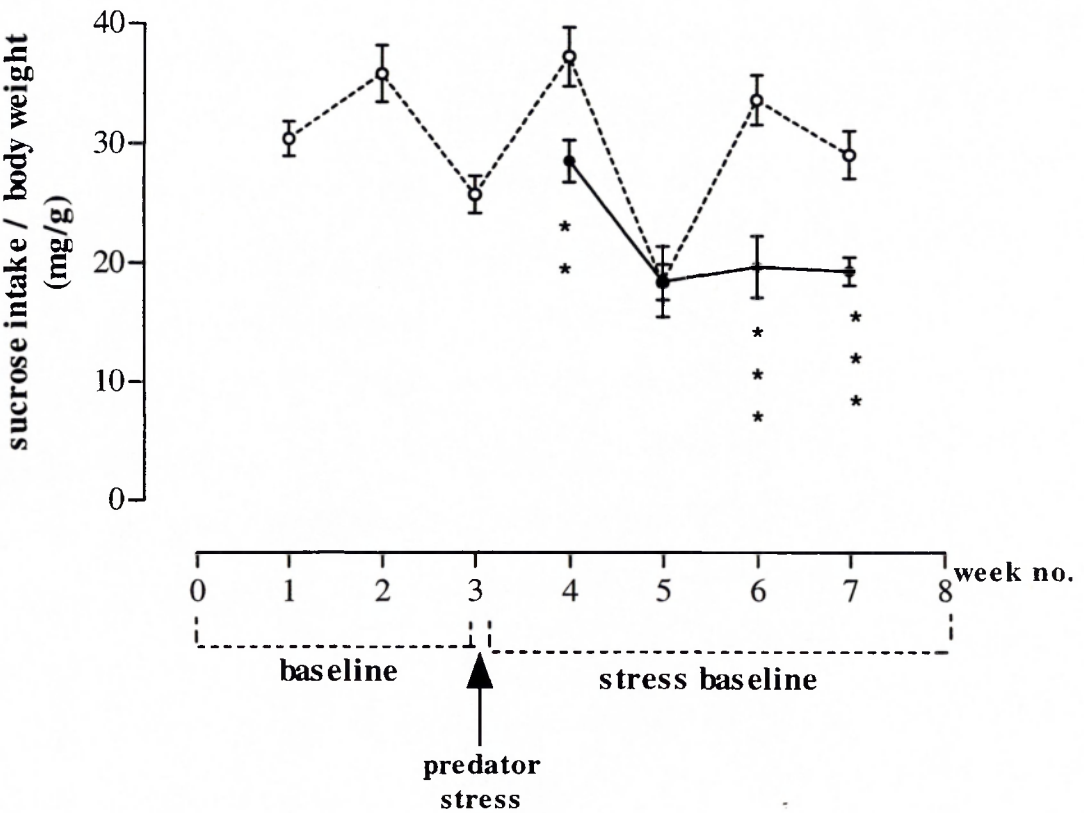
Effects of chronic exposure to predator stress and administration of vehicle (A), DMI (B) and paroxetine (C) on sucrose intake of BALB/c mice



Intake of a 3% sucrose solution by BALB/c mice was measured using one-bottle consumption tests over a 3 hour period following a 3-hour food and water deprivation period (see section 6.3.3.iii). Tests were conducted over 8 weeks (4 weeks before, and after the introduction of a predator stress stimulus). Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5mg/kg) began at week 8 for 28 days. Data are expressed as mean ± sem, see Appendix 6.3.C. Statistically significant differences were determined using ANOVA followed by Student's t-tests (GB-STAT v6.5) and are denoted by *p<0.05, ** p<0.01 or ***p<0.0001. ***Open circles = non-stressed group, closed circles = stressed group. ANOVA;*** *effect of stress – F (1, 329)= 20.35, p<0.0001; effect of treatment- F (2, 329)= 1.15, p= 0.32; effect of time- F (7, 329)= 2.72, p=0.009; interaction stress x treatment F (2, 329)= 1.94, p= 0.15; interaction stress x time- F (7, 329)= 1.47, p= 0.18; interaction treatment x time- F (14, 329)= 1.2, p= 0.27; interaction stress x treatment x time- F (14, 329)= 0.95, p= 0.5.*

Figure 6.3.3.iii. (3)

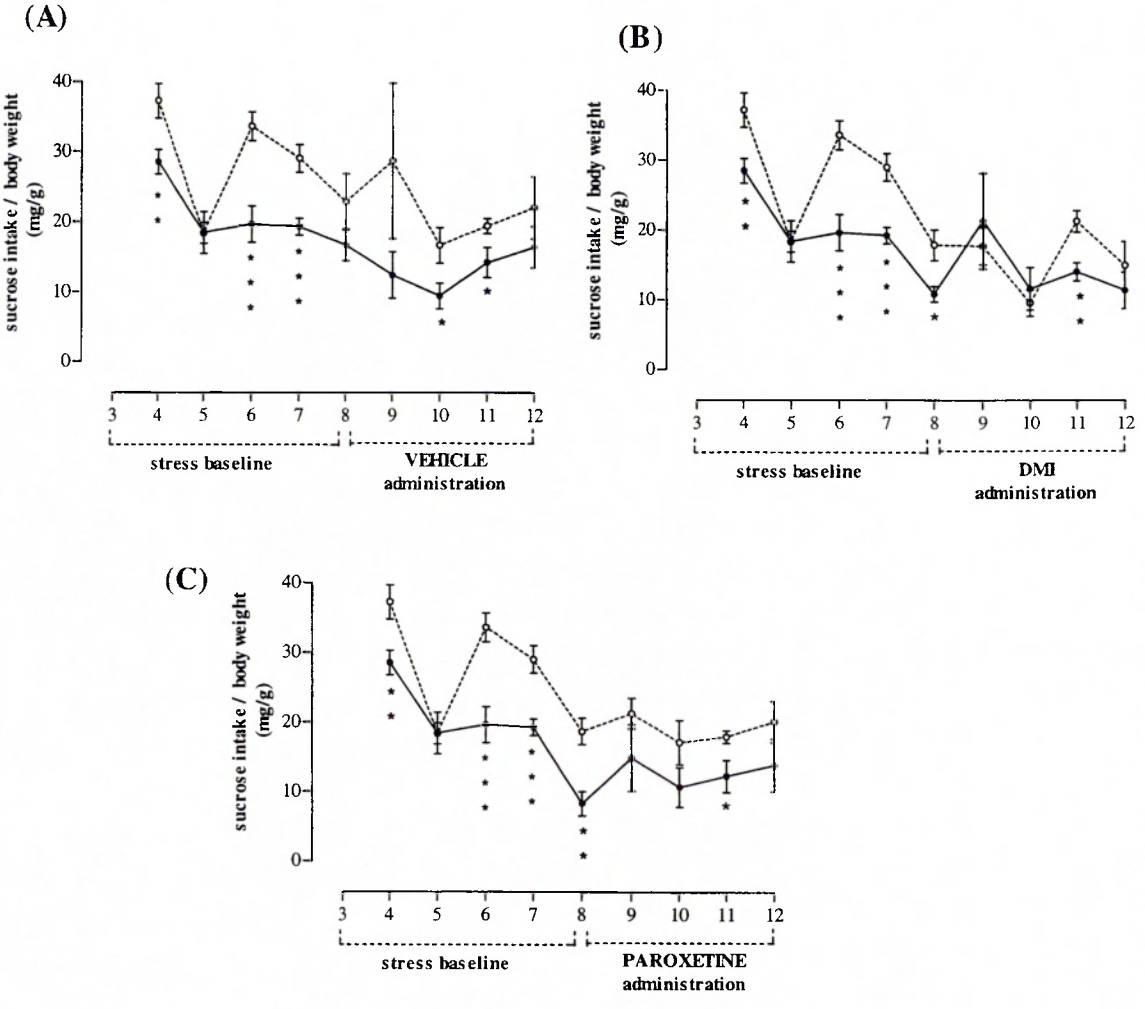
Effects of predator stress on sucrose intake/ body weight (mg/g) of individually housed BALB/c mice.



Sucrose intake / body weight (mg/g) of BALB/c mice (see section. 6.3.3.iii). Tests were conducted over 8 weeks before and after the introduction of the predator stress stimulus. Data are expressed as mean ± sem, see Appendix 6.3.D. Statistically significant differences were determined using ANOVA followed by Student's t-test (GB-STAT v6.5) and are denoted by ** $p<0.01$ and *** $p<0.0001$. ***Open circles = non-stressed group, closed circles = stressed group. ANOVA;*** *effect of stress – $F(1, 238)= 27.65$, $p<0.0001$; effect of time- $F(3, 238)= 15.16$, $p<0.0001$; interaction stress x time- $F(3, 238)= 4.19$, $p= 0.0065$.*

Figure 6.3.3.iii.(4)

Effects of chronic exposure to predator stress and administration of vehicle (A), DMI (B) and paroxetine (C) on sucrose intake / body weight (mg/g) of BALB/c mice



Sucrose intake / body weight (mg/g) of BALB/c mice (see section 6.3.3.iii). Tests were conducted over 8 weeks (4 weeks before, and after the introduction of the predator stress stimulus). Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5mg/kg) began at week 8 for 28 days. Data are expressed as mean ± sem, see Appendix 6.3.D. Statistically significant differences were determined using ANOVA followed by Student's t-tests (GB-STAT v6.5) and are denoted by *$p<0.05$, ** $p<0.01$ or ***$p<0.0001$. ***Open circles = non-stressed group, closed circles = stressed group. ANOVA;*** *effect of stress – $F(1, 203)= 22.7$, $p<0.0001$; effect of treatment- $F(2, 206)= 1.26$, $p= 0.29$; effect of time- $F(4, 203)= 3.1$, $p=0.017$; interaction stress x treatment $F(2, 203)= 0.71$, $p= 0.49$; interaction stress x time- $F(4, 203)= 0.31$, $p= 0.87$; interaction treatment x time- $F(8, 203)= 0.73$, $p= 0.66$; interaction stress x treatment x time- $F(8, 203)= 1.01$, $p= 0.43$.*

6.3.3.iv. Radioimmunoassay of plasma corticosterone concentrations

Non-stress and stress groups used to establish baseline values showed no differences in plasma corticosterone concentrations. Following commencement of drug/vehicle administration, plasma corticosterone levels were significantly higher in both stressed and non-stressed groups, as compared to respective baseline values. There were no significant differences in plasma corticosterone concentrations between stressed and non-stressed groups following 28 days of vehicle or drug administration (Table 6.3.3.iv). There were no significant effects or interactions of stress or drug treatment on plasma corticosterone concentrations following ANOVA.

Table 6.3.3.iv Plasma corticosterone concentrations (ng/mL) of BALB/c mice following chronic exposure to predator stress and 28 days of antidepressant administration.

	NON-STRESS	STRESS
Baseline	23.1 ± 1.8	21.4 ± 5.0

Drug treatment	**NON-STRESS**	**STRESS**
Vehicle	42.5 ± 5.0 *	32.3 ± 3.3 *
DMI	35.0 ± 5.6	45.8 ± 8.2
Paroxetine	42.3 ± 8.94	32.4 ± 2.8

BALB/c mice (n=6-8) were exposed to predator stress for 5 weeks following which they received the antidepressant drugs, DMI (15 mg/kg, p.o.) and paroxetine (7.5 mg.kg, p.o), once daily for 28 days. Animals were sacrificed after the 28 days of drug administration and blood samples collected for the radioimmunoassay of plasma corticosterone (see section 6.3.2.vii.). Values ranged from 19-79ng/mL non-stressed group; 12-70ng/mL stressed group. Data are expressed as mean ± sem. No statistically significant differences were determined using two-way ANOVA. Students t-tests revealed statistically significant differences which are denoted by * $p<0.05$ (as compared to respective baseline values). *ANOVA; effect of stress – $F(1, 43)= 0.45$, $p=0.51$; effect of time- $F(2, 43)= 0.19$, $p=0.83$; interaction stress x time- $F(2, 43)= 2.22$, $p= 0.12$.*

6.3.3.v. Specific ^{3}H-dexamethasone binding to mouse cytosolic CR

There were no significant differences in ^{3}H-dexamethasone binding to CR in mouse cortex taken from the non-stressed and stressed baseline groups (Table 6.3.3.v.A).

Cortical B_{max} values were not different from baseline non-stressed and stressed groups in vehicle treated non-stressed and vehicle treated stressed groups. B_{max} values in mouse cortex did not differ from vehicle treated groups following 28 days of DMI or paroxetine administration in both non-stressed and stressed groups. No significant effects or interactions of stress or drug administration were observed following analysis of variance.

No significant differences were found between transformed log K_D values in the cortex between any of the groups using ANOVA or Student's t-tests. ANOVA results showed no significant effects or interactions of stress or drug administration on ^{3}H-dexamethasone binding parameters to CR in mouse cortex (Table 6.3.3.v.A).

In the hippocampus, ^{3}H-dexamethasone binding to CR in the non-stress baseline group was not different to that in the stress group. No differences in B_{max} values were found using two-way ANOVA or Student's t-tests in any of the other groups or following chronic administration of vehicle, DMI or paroxetine (Table 6.3.3.v.B). No significant effects or interactions of stress or drug administration on ^{3}H-dexamethasone binding to hippocampal CR were observed following ANOVA.

There were no significant differences in protein values obtained in cortex or hippocampus using the Lowry method between any of the groups investigated (see Tables 6.3.3.v).

Table 6.3.3.v. ^{3}H-dexamethasone binding to CR in cortex (A) and hippocampus (B) taken from BALB/c mice following chronic exposure to predator stress and 28 days of antidepressant administration.

(A) CORTEX

Drug treatment	NON-STRESS			STRESS		
	B_{max} (fmol/mg protein)	K_D (nM)	Protein (μg/assay)	B_{max} (fmol/mg protein)	K_D (nM)	Protein (μg/assay)
Baseline	161± 20	1.62± 0.35	94 ± 7	193 ± 34	2.38± 0.55	78 ± 6
Vehicle	184 ± 7	2.96 ± 0.58	81 ± 85	195 ± 10	2.67 ± 0.45	85 ± 7
DMI	185 ± 13	2.27 ± 0.79	91 ± 4	167 ± 13	2.05 ± 0.51	89 ± 9
Paroxetine	163 ± 6 *	2.42 ± 0.63	72 ± 6	188 ± 11	2.52 ± 0.49	72 ± 4

(B) HIPPOCAMPUS

Drug treatment	NON-STRESS		STRESS	
	B_{max} (fmol/mg protein)	Protein (μg/assay)	B_{max} (fmol/mg protein)	Protein (μg/assay)
Baseline	142 ± 6	68 ± 8	172 ± 15	63 ± 7
Vehicle	139 ± 16	59 ± 8	146 ± 16	63 ± 5
DMI	143 ± 23	64 ± 7	144 ± 17	57 ± 6
Paroxetine	146 ± 13	62 ± 5	160 ± 13	66 ± 4

BALB/c mice (n=6-8) were exposed to predator stress for 5 weeks following which they received the antidepressant drugs, DMI (15 mg/kg, p.o.) and paroxetine (7.5 mg.kg, p.o), once daily for 28 days. Animals were sacrificed after the 28 days of drug administration and cortical and hippocampal tissue samples collected for the CR binding assay (see section 6.3.2.viii.). Values ranged from; CORTEX B_{max} - 91-239 and 108-273 fmol,mg protein (non-stressed, stressed groups respectively) / K_D - 0.27-5.81 and 0.62-6.1 nM (non-stressed, stressed groups respectively) / HIPPOCAMPUS - B_{max} - 72-223 and 86-221 fmol,mg protein (non-stressed, stressed groups respectively). Data are expressed as mean ± sem. Results were analysed using two-way ANOVA followed by Students t-test (for all vehicle/drug administered groups) and statistically significant differences are denoted by **p<0.05 –as compared to non-stressed vehicle-treated group. ***ANOVA (CORTEX CR B_{max}); effect of stress–F (1, 43)= 0.48, p=0.49; effect of time- F (2, 43)= 1.3, p=0.28; interaction stress x time- F (2, 43)= 2.52, p= 0.09. (CORTEX CR K_D); effect of stress–F (1, 43)= 0.055, p=082; effect of time- F (2, 43)= 1.3, p=0.3; interaction stress x time- F (2, 43)= 0.06, p= 0.94. ANOVA (HIPPOCAMPUS CR B_{max}); effect of stress – F (1, 45)= 0.3, p=0.59; effect of time- F (2, 45)= 0.26, p=0.77; interaction stress x time- F (2, 45)= 0.07, p= 0.93.***

6.4. Discussion

6.4.i. Body weights

Acute stress studies - Animals were not weighed over the acute stress studies, as these were too short for any changes to be observed.

Chronic stress and antidepressant studies - Body weights were generally lower in stressed animals as compared to non-stressed controls following introduction of the predator stress. Differences in body weights between stressed and non-stressed animals became significant in the vehicle treated stress group after 9-10 weeks of predator stress and when the dosing regime began.

Though body weights of mice exposed to the predator were lower than those of non-exposed mice, it seems that predator exposure could be classified as a mild stressor as it does not appear to have significant effects on body weight. Adamec & Shallow (1993) have also suggested that the lasting effects of rodent anxiety following cat exposure are mild, as they also found no changes in body weight in stressed animals. Reductions in body weight would be expected at all times if this stressor were a severe one.

Significant reductions in mouse body weights were observed only after the commencement of the dosing regime. It is possible that the dosing procedure itself may have been an additional stress inducing factor and, added to the effects of predator exposure, lead to reduced body weights in the stress group. This effect was prominently observed in the vehicle treated stressed group.

Body weights were also lower in predator exposed animals which had been given DMI or paroxetine over four weeks though these effects were not statistically significant suggesting that the antidepressant administration may be preventing weight loss due to

predator exposure or dosing, or both. DMI treated animals appeared to gain weight slower than those treated with paroxetine- this may be an effect of the appetite suppression properties of DMI.

It appears from these data that predator stress stimulus is mild, having insignificant effects on body weights. Predator exposure, in combination with the stress experienced during dosing, has a greater effect on body weights and the combination of these factors appears to be very stressful with regards to this parameter.

6.4.ii. Open field test

Acute stress studies - Locomotor activity in the open field arena of mice immediately, and 24 hours after exposure to the predator was not significantly different from non-exposed controls. Significantly reduced freezing behaviour was observed in the stressed groups as compared to controls (as demonstrated by decreased mobility latency). This observation may indicate the onset of a hyperactivity-like syndrome in animals acutely exposed to predator exposure.

No significant effects were observed in any of the other parameters measured in the open field test. Changes in rearing and grooming have been demonstrated in other studies (Blanchard *et al*, 1993) however these measurements were conducted in different arenas (hole board tests and plus maze) and it is difficult to compare them with our investigations and results.

Chronic stress and antidepressant studies – Ambulation scores were significantly lower in the stressed groups following several days of exposure to predator stress and four weeks of predator stress + vehicle administration. This may reflect reduced

activity suggesting anxiety-like behaviour in response to the stress of chronic predator exposure (Lister, 1990). This observation does not correspond with the higher ambulation scores observed in predator exposed animals in the acute stress studies. Freezing behaviour was also significantly increased in animals chronically exposed to predator stress (reflected by increased mobility latency as compared to non-stressed groups). These observations suggest a dissociation of locomotor activity responses following acute and chronic predator exposure. Acute predator stress appears to induce hyperactivity like syndrome in stressed animals whereas chronic exposure to the same stressor induces more immobility and freezing behaviour.

Alterations in either direction in the open field can be considered to reflect different types of stress. Hyperactivity in the open field is also a feature of the olfactory bulbectomised rat model of depression in which it is thought to result from a deficit in risk assessment and adaptation due to the malfunctioning of various neurotransmitter systems (see chapter 4). It may be that similar mechanisms have been triggered following acute exposure to predator stress. If this is the case, then our acute study data support the notion that exposure to the predator is having a stressful effect on the mice. Reduced activity in an open field environment has been suggested as being a useful index of anxiety (Lister, 1990) therefore it appears that chronic predator stress may be producing anxiety-like behaviour in the BALB/c mice.

Ambulation scores in stressed groups were not significantly altered in stressed or non-stressed groups following vehicle, DMI and paroxetine administration. Antidepressant administration did not exert any significant effects on the reduced activity of BALB/c mice following chronic predator exposure. Freezing time in chronically stressed animals was significantly reduced following 28 days of DMI and paroxetine

administration. However, this reduction was also observed following vehicle administration therefore cannot be attributed to antidepressant effects. These results are not consistent with those of Blanchard *et al* (1993) who demonstrated that pre-treatment for 15 days with imipramine reduced anti-defensive behaviours (eg. reduced freezing, proxemic avoidance) of rats when exposed to a cat however, this investigation was conducted in a very different manner from our own and comparisons are made difficult.

Defecation of BALB/c mice exposed to predator stress for several days was lower than that of unstressed controls. This effect was more pronounced in the vehicle treated group following the introduction of the dosing regime; though there were several changes in the non-stressed group. Stress experienced by the mice whilst dosing may have some effect on defecation measures; maybe more so in the non-stressed control group than those exposed to predator stress. Administration of DMI and paroxetine brought the defecation counts in stressed animals more in line with non-stressed values.

It could be argued that some of the effects observed in these studies may be attributed to some degree of habituation effect of the mice to the open field arena. A decline in exploratory behaviour and activity is generally observed as tests are repeated and the novelty of the test conditions disappears. Some studies have demonstrated day-to-day reductions in the activity of rats tested repeatedly on an enclosed plus maze but no such decline is observed in the open arm maze (Montgomery, 1954; Lister, 1990). Experiments by Montgomery (1954) demonstrated that novel stimulation evokes both the exploratory drive and the fear drive thus generating approach-avoidance conflict behaviour in rats. The strength of the fear drive decreased with time of direct exposure

to the stimulus. Representation of the novel stimulation following a period of non-exposure resulted in a spontaneous recovery of fear.

There are few valid arguments as to why these findings should not also apply to mice. In line with these suggestions, the open space component of the OFT, in addition to the frequency of testing (each OFT was 2 weeks apart) and relatively short testing time (5 minutes), may confer some degree of resistance to habituation to this test.

The open field arena constitutes a stressful, novel environment for the subjects however, the purpose of using this method in our investigations has not been solely to mount an additional stress challenge on the subjects but to obtain a 'snapshot' measure of activity over normal, stress and drug administration periods. In both acute and chronic studies, mice were exposed to the open field test on more than one occasion so none of the values recorded, even in the acute study were measured during a first test in the open field arena. Also, in the event of any behavioural habituation occurring to the open field arena, effects would be observed in both unstressed and stressed groups alike; this has not been found to be the case in our experiments.

Blanchard *et al* (1993) demonstrated behavioural changes consistent with a high level of risk assessment (scored mainly by stretch-attend posture towards the stimulus) and fear/anxiety in rats on presentation of cat odour. The acute effects of predator exposure in our studies using the open field are consistent with increased risk assessment behaviour, which are associated with reduced anxiety.

Some studies have however reported reduced exploration behaviour (open-arm entry in the plus maze) and risk assessment behaviour of rats (activity and rearing in hole board

tests) as lasting effects following cat exposure (Adamec *et al*, 1993a; 1998). These investigations involved a single exposure to the predator and measured various behaviours up to 21 days after the exposure. Reduced risk assessment behaviour is associated with increased anxiety therefore it appears that the BALB/c mice in our study may be experiencing high levels of anxiety following chronic exposure to predator stress.

6.4.iii. Sucrose tests

Many alterations in various parameters have been found following exposure to predators and/or their odour in rats (see section 6.1). Numerous studies have also successfully employed sucrose intake tests to measure reward behaviour and anhedonic responses in rats and mice following CMS procedures (Muscat & Willner, 1992; Willner *et al*, 1996). The aim of these experiments was to investigate any possible changes in sucrose intakes of BALB/c mice following acute or chronic exposure to a predator.

Acute stress studies - Consumption of a 3% sucrose solution was significantly reduced immediately following acute exposure to predator stress. These data support the idea that exposure to a predator may be inducing some kind of stress response in BALB/c mice. The reduction of sucrose intake over sub-chronic and chronic periods of time has been compared to anhedonic effects in previous models of stress and depression (see section 5.1.) and the results of experiment 1 may support the hypothesis of an anhedonic response following predator stress. However, the acute time course of this effect also raises the possibility that the reduction of sucrose intake may be due directly to a short-term fear/anxiety response. The results of experiment 2 appear to support this possibility as, 24 hours after the predator exposure, there were no differences in sucrose

intake between control and stressed animals, thus suggesting that single exposure to predator stress may not cause long-lasting effects in sucrose consumption tests.

Blanchard *et al* (1993) have also demonstrated a reliable reduction in eating frequencies, levels and duration in groups of rats following a single exposure to a cat suggesting that predator exposure may act to suppress feeding behaviour.

Chronic stress and antidepressant studies – Sucrose intake was significantly reduced (albeit erratically) in BALB/c mice following repeated exposure to a predator. Decreases in sucrose intake were seen after the first exposure (in keeping with the findings of the acute stress study – experiment 1) and are generally maintained after predator exposure for approximately 4 weeks. It appears also that sucrose intake is reduced, again erratically, both in stressed and unstressed mice following the commencement of the dosing regime suggesting that this may be an additional stressful component of the study with regards to this particular measure. Sucrose intake remains lower in the vehicle treated stress groups than in non-stressed controls indicating that the predator stress stimulus is still exerting some effect.

The long-term administration of DMI and paroxetine appeared to bring sucrose intake values in stressed and non-stressed mice closer together. However, this effect was, on closer inspection due to reductions in intake in the non-stressed control groups rather than a restorative effect of antidepressant administration on decreased sucrose intakes following predator stress. These data are partly inconsistent with the reported restoration of chronic stress-induced reduced sucrose intake following antidepressant administration in mice (Monleon *et al*; 1995) and rats (Muscat & Willner, 1992a; 1992b; Papp *et al*, 1994a; 1994b) following the administration of imipramine in several

CMS studies. Our results are also inconsistent with those of D'Aquila *et al* (1997a) and Muscat *et al* (1992), who found that chronic administration of fluoxetine gradually restored sucrose intakes in mice and rats stressed using the CMS procedure. The reversal of CMS induced anhedonia has also been demonstrated following administration of the antidepressant compounds, maprotiline and flibanserin in laboratory animals (Muscat *et al,* 1992; D'Aquila *et al,* 1997a). The variability of the sucrose test data however made it very difficult to evaluate the time course of DMI or paroxetine effects on sucrose intake.

Though numerous investigations using CMS have reported reductions in sucrose intake as a measure of decreased reward, the reliability of sucrose intake as a measure of anhedonic status has always been a controversial issue (Forbes *et al,* 1996; Harris *et al,* 1998). There appears to be some conflict regarding the reliability of this measure (D'Aquila *et al,* 1997a; 1997b; Willner, 1997) and sucrose intake has been found to be rather erratic in various studies. It is possible that this parameter could be influenced by a variety of factors unrelated to hedonic tone (Willner; 1992).

It has been argued that the reduction in sucrose intake observed in many CMS studies may be due to an accompanying loss of body weight, dehydration state and/or calorie restriction following long food and water deprivation periods (Forbes *et al,* 1996; Harris *et al,* 1998). In applying these criticisms to our studies, it is agreed that body weights are lower in stressed groups however, this is not a significant effect and the size of this effect (maximum ~5%) is minimal when compared to the size of the effects on sucrose consumption (25%-week 3, 49%-week 4, 44%-week 5, 48%-week 6, 34%-week 7 and 40%-week 8). Sucrose intake data, when expressed as mg intake/g body weight (Figures 6.3.3.iii.(3) and (4)) also generally demonstrated reduced intake

following stress. The general lack of significant differences in sucrose intakes following DMI and paroxetine administration may have resulted from reduced sucrose intakes in the non-stressed control groups rather than "restored" intakes in the stressed groups. This is inconsistent with the findings in CMS studies (Willner *et al*, 1992; 1996; D'Aquila *et al*, 1997).

Reductions in sucrose intake may be also attributed to a general decrease in non-specific consummatory behaviour. Water intake in stressed and non-stressed animals did not differ throughout this particular study. Food intake (as measured in a parallel study, Theodorou & Bate - personal communication) was found to be gradually reduced in both groups throughout the study.

It could also be argued that reduced sucrose intake may not model an anhedonic response as, though the animal's consumption of a sweet sucrose solution may have altered, this may not necessarily be indicative of a loss of preference. In our studies, 2-bottle tests were abandoned (section 5.5) as they gave similar results to, and did not provide any more additional information than the more convenient 1-bottle test therefore preference for sucrose was not recorded in this study. Water intake was measured at various points and remained constant (and lower than sucrose intake in no-stressed animals) throughout the investigation. These data also concur with results of experiments following CMS in rats (Muscat *et al*, 1992a; 1992b).

The data obtained in these experiments shows that exposure of mice to the odour and visual contact with a predator is effective in reducing the intake of a 3% sucrose solution (which has been shown to be preferred by the mice over water – section 5.2.4). This suggests the presence of decreased reward sensitivity and induction of an

anhedonic state in mice chronically exposed to a predator. No reversal of this effect following chronic antidepressant treatment was observed in this study.

6.4.iv. Plasma corticosterone concentrations

Acute stress study – No statistically significant differences were observed in plasma corticosterone concentrations following acute exposure to predator stress though corticosterone concentrations were elevated by 39% immediately after exposure. Previous studies have reported increased rat plasma corticosterone concentrations in response to predator odour (File *et al,* 1993a; Lu *et al,* 1998; Tanapat *et al,* 1998) and have suggested that this effect may reflect an acute fear/anxiety response that appears to fade with time after exposure (File *et al,* 1993a). Lasting effects of a single exposure to a predator on plasma corticosterone are found to vary with studies reporting increased corticosterone for up to one week after exposure in Hooded rats but not in Wistar rats (Adamec *et al,* 1997a; 1997b). Acute exposure to a predator in our studies however did not produce these effects.

Chronic stress and antidepressant study - Plasma corticosterone concentrations were not significantly affected by chronic exposure to predator stress. No effects of chronic predator stress were observed in the plasma corticosterone concentrations of the baseline non-stress and stress groups. However, these observations are in line with studies conducted by File *et al* (1993a) which found that the corticosterone response to predator odour stress in rats diminishes with increased number of exposures to the predator. The corticosterone response represents the 'disturbance' component of the phobic reaction of rats to predator odour and is found to habituate more readily in rats than the behavioural 'avoidance' response (File *et al;* 1993a). Bearing this in mind (and also unpublished observations by Karen Mellowdew, Institute of Psychiatry that odour

alone does not appear to induce significant stress in mice), the mice in these experiments were also exposed to visual predator stress in an attempt to make the predator stimulus more stressful. The results of our studies indicate that the protocol used for predator stress may not be sufficient to elicit a corticosterone response in BALB/c mice. Any possible intermediate responses may have been overlooked as plasma corticosterone was only measured at the end of the investigation and may be subject to rapid habituation, not only to the odour but also to visual contact with the predator.

The largest differences in corticosterone levels were observed between the baseline non-stress/stress groups and the vehicle treated non-stress/stress groups suggesting that the dosing procedure itself may be inducing some degree of stress in all of the animals. The similarity of plasma corticosterone concentrations in the non-stressed vehicle treated group and stressed vehicle treated group indicates that exposure to predator stress in combination with the stress of dosing is not a factor leading to elevated corticosterone levels in this group. The degree of handling experienced by the animals may also be a factor affecting the corticosterone response as animals were regularly handled throughout the study.

There were no overall significant effects of chronic antidepressant treatment on plasma corticosterone in this study.

6.4.v. Specific ^{3}H-dexamethasone binding to cortical and hippocampal CR

Acute stress studies - No changes were observed in any of the parameters of specific ^{3}H-dexamethasone binding to CR in cortex or hippocampus following acute exposure to predator stress. It is likely that the stressor may be too acute to cause receptor changes. Also, the lack of any significant increase in plasma corticosterone concentrations may not be sufficient to induce changes in receptor occupancy in the stressed animals.

Chronic stress and antidepressant studies – There were no significant changes in specific ^{3}H-dexamethasone binding to CR in either cortex or hippocampus following long term exposure to predator stress. This indicates that there is no mechanism acting to down-regulate CR in these brain regions following chronic predator exposure or that this stimulus may not be stressful enough to produce a clear reduction in CR numbers. It may also be possible that the presence of endogenous corticosterone in the system at the time of sacrifice is confounding data and reducing the CR signal being measured by the assay. However, the lack of effects observed in plasma corticosterone indicates that this is not likely.

The dosing procedure appeared to have no effect on the B_{max} of ^{3}H-dexamethasone binding in the cortex or hippocampus. No effects of chronic dosing with DMI were observed in non-stressed or stressed animals. Treatment with paroxetine in the non-stress control group significantly reduced ^{3}H-dexamethasone binding to cortical CR as compared to the non-stressed vehicle treated group. This observation agrees with the results of the naïve study using paroxetine (Chapter 3) in which chronic administration of paroxetine down-regulated CR in rat cortex.

These data may reflect habituation to the stressor as Zangrossi & File (1993) have demonstrated a dissociation of behavioural and corticosterone responses to cat odour in rats. Though behavioural responses are maintained following repeated exposure to a predator (File *et al*, 1993a), corticosterone responses rapidly habituate. It follows that no changes will be seen in CR binding as no dysfunction of the HPA axis is observed in this paradigm.

6.4.vi. General

Though several previous studies have demonstrated a lasting effect of predator exposure (Blanchard *et al*, 1993; Adamec *et al*, 1993; 1998), few have investigated the effects of repeated predator exposure on any particular measures. One such study, conducted by Pico-Alfonso *et al* (1998) demonstrated no changes in sucrose intake or plus maze behaviour following 15 days of exposure to predator stress. The results of this are consistent with our data and suggests that 15 days of exposure may not be enough to produce stress effects.

More methodological considerations of this investigation involve possible criticisms of housing conditions, the effects of circadian fluctuation in measures and more prominently, the effects of food and water deprivation. Some investigations have indicated that social isolation is associated with increased food consumption and anxiety in rats (Scalera *et al*, 1992). Mice however, have a different social organisation and are found to be behaviourally isolated in natural situations in that they do not tolerate male conspecifics in their territory. This situation may be different in the case of laboratory mice however, Moles *et al* (1995) found no differences in sucrose intake measures in individually housed mice.

It can be argued that more accurate measures of activity and intake could be obtained in the dark phase of the activity cycle as mice are nocturnal animals. However, our preliminary experiments (Chapter 5) demonstrated there were no significant differences in measures taken in the light or dark phase.

The issue of food and water deprivation is an important one; however it appears that the changes observed in our studies are not directly attributed to body weights, dehydration status or calorie restriction. Food deprivation however, is also found to affect a number of other factors. Insulin has been demonstrated to target the striatum and regulate the activity of the dopamine transporter (the major mechanism of DA clearance). As a result of food deprivation, Patterson *et al* (1998) reported decreased DA transporter mRNA levels in the striatum. Though these studies involved long periods of fasting for rats, a regular restriction of food intake may have some impact on DA systems, which are involved in the reward behaviour being measured by sucrose testing. Restriction of food intake has also been reported to increase corticosterone concentrations in rats and also produce a dysregulation of 5-$HT2_C$ receptor mRNA and abolish the normal diurnal variation in hippocampal GR and MR (Holmes *et al*, 1997).

Neural mechanisms of anhedonia have been investigated using CMS, place preference conditioning and intra-cranial self-stimulation (ICSS). The mesolimbic DA projection from the ventral tegmental area (VTA) to the nucleus accumbens is thought to play a crucial role in mediating the behavioural effects of rewards. Behavioural effects induced by CMS have much in common with the effects of DA receptor antagonists such as SCH-23390, pimozide, sulpiride and raclopride. Primary features of these include the selective suppression of dilute sucrose intake/preference, attenuation of place preference conditioning and an increase in the electrical threshold for ICSS

through VTA implanted electrodes (Willner *et al*, 1992). Changes in sensitivity to rewards are likely to be mediated post-synaptically following subsensitivity of D_2 receptors in the nucleus accumbens (Towell *et al*, 1987; Muscat & Willner, 1989; Muscat *et al*, 1992b, Papp *et al*, 1994b), this effect being secondary to a prolonged and persistent overexposure to increased DA release. There is also strong evidence to suggest that chronic antidepressant administration may reverse anhedonia by potentiating DA transmission. Therapeutic responses to antidepressants are found to be reversed by DA receptor antagonists, all of these reducing sucrose intake only in antidepressant treated, stressed animals. Muscat *et al* (1992b) also reported that the therapeutic effects of fluoxetine and maprotiline, which act mainly as 5-HT and NA reuptake inhibitors, were reversed by acute administration of raclopride suggesting that sensitised D_2 receptors in the nucleus accumbens may provide a common mechanism of action of antidepressant drug actions on anhedonic behaviour.

Changes in 5-HT are associated with many, but not all, anxiogenic and stressful situations. Increased GABA release, with accompanying decreases in uptake, have been reported in cortical and hippocampal rat brain slices following cat exposure. Changes in release and uptake of GABA after novel environment exposure were in the opposite direction. (File *et al*, 1993b). Hippocampal 5-HT concentrations did not appear to be significantly affected by cat exposure and were found to be time-dependent and region specific, as were measurements of 5-HT concentrations following novel environment exposure. This investigation suggests that anxiogenic responses may not always be associated with altered 5-HT levels as traditionally thought.

It appears, from our data and also previous studies, that the nature of the effects of predator stress may be dependent on the behavioural measure. The acute nature of the

stressor may indicate a fear response to this stressor on measures of open field activity, sucrose intake and also plasma corticosterone concentrations. As alterations in all/many of these parameters are expected in an anxiety model, it is not clear whether this stressor is producing anxiety or stress/depression effects. The changes seen in sucrose intake are indicative of anhedonic effects following chronic predator exposure, which reflects a core symptom of melancholia; however the lack of antidepressant effects implies that this paradigm may not be responsive to antidepressant compounds. Depressed activity following chronic predator exposure may reflect a core symptom of melancholia (in which motor activity can be either increased or decreased) or a typical anxiety pattern. The lack of significant effects on biochemical parameters indicates that, though this paradigm may model some of the behavioural aspects of stress and anxiety, it does not model the endocrinological or biochemical symptoms to any great degree. The nature of the stressor plays an important role in the activation of stress responses resulting in various symptoms being displayed (Herman *et al*, 1997). Many of the alterations observed in this paradigm suggest that it may be a useful model of post-traumatic-stress disorder (PTSD) and related anxiety.

CHAPTER 7

DISCUSSION

Discussion

Major biological manifestations of major depression include alterations in limbic/hypothalamic centres that govern food intake, libido, circadian rhythms and hormone secretion. The preceding experiments used these observations as their starting points in attempting to elucidate the mechanisms that underlie antidepressant activity. Consistent defects have been observed in corticosteroid regulation of the HPA axis in both hypercortisolaemic, depressed patients and stressed animals, which appear to be normalised following antidepressant treatment. The main objectives of the present investigation were to determine whether stress syndromes and antidepressant administration in animals were associated with alterations in corticosteroid receptors/secretion and if CR alterations may form a common pathway for the mechanism of action of various antidepressant drugs.

7.1. Methodological considerations

^{3}H-dexamethasone binding assay; CR radioligand binding studies have used various ligands (^{3}H-corticosterone, ^{3}H-dexamethasone, ^{3}H-RU28362, ^{3}H-aldosterone) for measuring CR – and dextran coated charcoal, gel filtration or centrifugation as separation methods (Luttge *et al*, 1984; Reul & de Kloet, 1985; Reul *et al*, 1993; Budziszweska *et al*, 1994b). Previous investigations have utilised various radioligands to measure CR including ^{3}H-corticosterone (Jacobsen *et al*, 1993), ^{3}H-aldosterone (Vedder *et al*, 1993) and ^{3}H-RU28362 (Reul *et al*, 1987a; 1987b). In our studies, ^{3}H-dexamethasone was used as a radioligand allowing us to measure CR in various brain tissues and also the thymus. The interaction between this ligand and the binding site in our experiments was found to be a saturable, simple bimolecular reaction that follows the law of mass action, thereby fulfilling the fundamental criteria of receptor binding analysis. The use of the rapid vacuum filtration method of separation of bound and free

ligand was also supported by the observation that the values obtained in our studies agreed closely with those obtained in other laboratories (Szuran *et al*, 1997); and also with studies obtained in our own laboratory using column chromatography (Rosser *et al*, 1995). The CR binding assay may be subject to the criticism that it is a rather sensitive assay that has the potential to be affected by numerous external factors. In our laboratory however, this assay has proven to be consistent over the years and the results of the studies conducted over the duration of this thesis have not been subject to large fluctuations over time.

Attempts to clarify the status of CR involvement with stress/depression and/or antidepressant action have mainly relied on the use of adrenalectomised animals for *in vitro* studies (Sapolsky *et al*, 1984a; 1984b; Reul *et al*, 1993; 1994; Budziszweska *et al*, 1994a). Our data was generated using adrenally-intact animals which contained endogenous circulating corticosterone at the time of death. Therefore many precautions were taken in order to ensure that basal corticosterone concentrations remained within the normal range with minimal fluctuations.

In order to obtain valid data pertaining to plasma corticosterone concentrations, it was important to use controlled conditions throughout. Any deviations from normal light/dark cycles, noise, light intensity and temperature may have generated the possibility of altering basal hormone levels, as well as inducing shifts in normal circadian rhythms. The circadian pattern of corticosteroid secretion also required samples to be collected at the same time each day. Animals were also minimally disturbed at all times to reduce the risk of stress-induced corticosteroid secretion. The levels of circulating corticosterone at the time of sacrifice corresponded with a 70-90% occupation of type I MR indicating that most of the CR being measured in our

investigations were type II GR. This conclusion was supported by the demonstration of a GR-like competition profile and similar binding parameters were obtained in saturation experiments using the selective GR agonist RU28362 to define specific binding.

Behavioural investigations; The subjective nature of these and the lack of standardised behavioural testing procedures across laboratories makes it difficult to compare data from various studies. Optimisation of testing procedures (as described in Chapter 5) for our own studies ensured that protocols were consistently observed. The species of animals used also appears to greatly impact any behavioural measurements (Capeless & Whitney, 1995; Logue *et al*, 1997). As the use of behavioural models and transgenic animals in the development of psychiatric disorders increases so does the importance of choosing animals with the appropriate genetic background, again, an issue raised by the lack of standardisation of protocols. Discrepancies between studies may be attributed to numerous possible variations in animal species used, the testing protocols and the receptor binding assays.

Depressed patients exhibit an altered timing of rest/activity. It may be useful, in future studies, to constantly monitor activity in animal model investigations instead of obtaining 'snapshot measures', as animals with HPA disorders are more likely to display altered circadian rhythmicity (maybe also incorporating hyperphagia- and hypersomnia-type syndromes).

7.2. Corticosteroid receptors and antidepressants

There is clear evidence to suggest that antidepressant compounds may act via the regulation of corticosteroid receptors to correct the defective feedback observed in stress/depression. Several investigations have suggested that CR and/or CR mRNA are down-regulated following stress (Sapolsky *et al*, 1984a; Young *et al*, 1990; Brooke *et al*, 1994a; Gomez *et al*, 1996; Gomez *et al* ,1998; Kitraki *et al*, 1999) yet few have investigated any potential normalisation of this deficit following chronic administration of various antidepressant drugs. Suggestions that up-regulation of CR may comprise a common mechanism of action for the different classes of antidepressant treatments through which therapeutic efficacy of these compounds is achieved, have been derived primarily from work in adrenalectomised, unstressed animals (Reul *et al*, 1993; 1994; Budziszweska *et al*, 1994a).

Our studies using naïve animals, indicated that the antidepressants DMI, paroxetine and venlafaxine may have different effects on CR in various brain regions following long-term administration in adrenally-intact rats. Inconsistent results were observed, with chronic administration (14 days) of DMI inducing increases in cortical CR; paroxetine inducing decreases in CR in the cortex and thymus following 14 and 28 days of administration, respectively, however an increase in striatal CR was observed after 7 days; venlafaxine had no consistent effects on CR in any of the regions investigated.

Previous studies have reported increases in hippocampal CR/CR mRNA following long-term administration of tricyclic antidepressants or moclobemide – these studies were conducted in adrenalectomised animals thus possibly rendering CR in this region more sensitive to fluctuations induced by antidepressant administration. The observed neuroanatomical specificities of the antidepressant effects in our studies could possibly

also relate to differing monoaminergic innervations of theses areas which may indirectly be affecting CR in these regions. In our studies, any changes have been observed primarily in the cortex and not in the hippocampus, suggesting a different mechanism may be active in the presence of endogenous ligand. The data obtained may have been confounded by the presence of endogenous corticosterone in the system at the time of assay as previous investigations used adrenalectomised animals only. However, the procedure of adrenalectomy itself can also interfere with the status of the HPA axis (see section 3.5) and though, in our studies, we may have been measuring only ~70-90% of the total CR population, this was sufficient to enable reflections of any changes in numbers of receptors following various procedures.

Previous investigations have not always used the same radioligand to measure CR; Budziszweska *et al* (1994b) used ^{3}H-corticosterone whereas Reul *et al*, 1993; 1994 have used ^{3}H-dexamethasone and ^{3}H-aldosterone in their experiments – these variations in CR measurement methods may also contribute to observed differences in results.

The lack of a consistent up- or down regulation of CR in our studies may also be attributed to the doses of antidepressants used as these differed between previous studies and our own, as did the route/method of administration. Future experiments may benefit from establishing dose-response effects of antidepressants and also the measurement of type I MR in these samples.

Our investigations also displayed no significant effects of stress or antidepressants on plasma corticosterone concentrations which may have been more accurately detected (in conscious animals) if we had used in-dwelling catheters to collect serial samples

rather than the trunk blood method. Serial sampling greatly increases the information obtained and also the investigative power of neuroendocrine challenge testing which could be used in future experiments of this nature.

The involvement of various neurotransmitter systems with HPA axis regulation is well documented (Dinan, 1996a; 1996b; Tritos *et al*; 1999). It is not clear however whether the HPA abnormalities observed in depression occur as a secondary result of primary neurotransmitter alterations or vice versa and arguments have been put forward for both mechanisms (Budziszweska; 1994a, Dinan; 1996b). Many investigations have demonstrated the responsivity of HPA axis elements to modulation of neurotransmitter levels and it has become clear that the relationship between monoamines (especially catecholamines and 5-HT) and the HPA axis is extremely complex with multiple levels at which the systems interact with each other. Other neuroactive peptides synthesised by the brain are also affected by the administration of antidepressants (CRF, BDNF, etc...) and these may also be involved in the cascade of events leading to HPA normalisation in depression.

Direct effects of antidepressant action on the HPA axis are also firmly established. It has been suggested that antidepressants may help to maintain CR function in NA and 5-HT containing neuron-containing cell groups, eg. the hippocampus (Holsboer & Barden, 1996) via their acute affects on neurotransmitter concentrations. None of the CR alterations in our studies were observed in the hippocampus therefore did not consolidate these suggestions.

7.3. Animal models of stress/depression

The role of animal models of depression may be considered as a critical interface between basic behavioural neuroscience and the clinic. In this respect, the preceding experiments used epidemiological, behavioural and endocrine correlates found in patients suffering from stress/depression as their starting points with attempts to reproduce similar syndromes in laboratory animals.

The OB rat, appearing to share many of the characteristics observed in stressed/depressed patients (Table 1.6.1.i) that are normalised by chronic antidepressant administration, was first investigated. All antidepressant compounds used in our studies normalised OB induced hyperactivity in the open field demonstrating that this parameter is replicable from study to study and reliable for use in the screening of compounds for antidepressant activity in the OB rat. These results were very consistent with the results of numerous previous investigations (section 4.1) Receptor binding studies failed to show any CR deficits in the bulbectomised rat suggesting that the OB rat does not model the dysfunctional corticosteroid feedback characterised by CR down-regulation observed in many depressed patients.

Time course studies, similar to those conducted in naïve animals were conducted in sham operated animals following the analysis of results from experiments that demonstrated that sham-operated controls are very similar to un-operated controls. The results of these investigations showed no consistent alterations in hippocampal CR following chronic administration of DMI, citalopram and milnacipran. These observations must also be tempered by the fact that CR binding was only tested at one dose of each of the antidepressants; a dose-response experiment may be useful here. Venlafaxine however did increase CR binding following 28 days of administration

displaying some differences between these data and those obtained in the naïve study and indicating that sham-operated rats may not be suitable for use as a control animal. The actual doses of antidepressants given in each of the investigations also differed however, providing more potential variation in experimental design.

Several of the classical alterations associated with the depressive syndrome in animal models however have not yet been observed in the predator stress paradigm. Investigations being conducted in this area are still in early stages however and the hypothesis that this behavioural paradigm may constitute to some degree, an animal model of depression/anxiety is recent. Overall responses of rats and mice to predator exposure indicate that this stimulus is a stressful one (see section 6.1). Responses to this stressor also indicate a high level of anxiety-like behaviour in animals exposed to predator stress. Some of the changes observed in this paradigm can be compared to those observed in human stress situations and also in a number of various other putative models of depression and anxiety.

Our predator stress studies showed some conflicting data with open field hyperactivity being observed following acute stress; this was reversed following chronic stress. No antidepressant effects were observed on open field activity. The open field arena is an established method by which activity levels are measured in laboratory animals. In attempting to simulate the psychomotor agitation or retardation observed in depressed patients, measurements in animals were made difficult by the demonstration of both increased and reduced activity following stress. However it is generally thought that reduced activity levels reflect more of an anxiety-like state (Lister, 1990; Adamec *et al*, 1993a) indicating that the predator stress paradigm may be more useful in the study of anxiety.

Sucrose intake was reduced following acute and chronic predator stress however the variability of the data made it difficult to evaluate accurately the effects of antidepressant administration on this parameter. Given the difficulties encountered in attempting to establish a sucrose intake baseline and recent controversy surrounding the issues of reliability of sucrose intake measures in stress models (Willner, 1997a; 1997b; Weiss, 1997; Moreau, 1997), the inconsistency of these results may be warranted.

The validity of using anhedonia as a measure of the 'loss of pleasure' experienced is also questionable. Anhedonia is found to occur to some degree in over 50% of psychiatric patients regardless of diagnosis (Silverstone, 1991- cited in Argyropoulos & Nutt, 1997). However, it has been suggested that anhedonia in chronic stress studies may result from motor retardation (Nelson & Charney, 1981, Argyropoulos & Nutt, 1997). The dopaminergic reward system (ascending from the mesencephalon to the ventral striatum/nucleus accumbens) is functionally and anatomically closely connected to the nigrostriatal extrapyramidal pathways therefore a dysfunction of both in psychiatric disorders may lead to both anhedonia and motor retardation. This must be kept in mind when looking at the predator stress studies as both behavioural syndromes were observed.

As with the OB rat, mice subjected to predator stress also did not display any alterations in corticosterone output and/or CR binding parameters, again indicating that defective CR regulation was not being modelled in this stress-based animal paradigm.

The lack of CR effects in the animal models investigated suggest that more work may need to be conducted along these lines. The predator stress clearly needs further behavioural, neurochemical and endocrinological characterisation and investigation.

HPA axis changes in both of the models used may be more pronounced following a stress challenge prior to sacrifice superimposed on the chronic stress administered. The predator stress, though ethologically sound, does not appear to be severe enough to cause changes in the parameters being measured. It is possible that the use of different predators (eg. cats or ferrets) may be more effective. However in using external stressors to induce stress syndromes in laboratory animals, one issue that has always caused concern is that of severity of the stressors utilised. Many behavioural and physiological alterations have been reported when stressors are severe (eg. immobilisation stress – Katz *et al*, 1981; 1982a; 1982b; Bonaz *et al*, 1998; Howell *et al*, 1998, Karandrea *et al*, 2000; Chowdhury *et al*, 2000) or caused by lesioning (Chapter 4). However in attempting to reproduce stress syndromes resulting in an animal model for depression, many investigations have preferred the use of milder, (Willner *et al*, 1991a; 1991b; 1997) or ethologically relevant stressors (Zangrossi *et al*, 1992a; Adamec *et al*, 1993; Chapter 6) which appear to provide a more realistic method of stress induction. This has certainly been the objective of the experiments presented in Chapter 6.

7.4. General

Although the original biochemical hypotheses of depression envisaged a single transmitter disorder, subsequent neuroscientific developments have highlighted the complex inter-relations between various systems (Rossby *et al*, 1994). This alone suggests that the physiological processes associated with depression may occur in various stages which may involve different systems at different times. This could provide some information regarding the large variety of depressive symptoms and why they are not uniformly experienced by all patients. It could be that seemingly

contrasting experimental findings may simply reflect different types or stages of depressive episodes.

There is much evidence for the existence of interactive communication between the CNS, endocrine and immune systems (Connor, 1998; Elenkov *et al*, 2000). Regulation of the immune response is partly controlled by the experience of stress, which appears to play a prominent role in depression aetiology. Glucocorticoids also mediate immunosuppression – a mechanism thought to have evolved in response to the fight/flight reaction. As with corticosteroid secretion, this is advantageous acutely but when chronically activated may have debilitating effects. It is quite possible that the immunological alterations observed in depression also occur as a result of excessive activation of the HPA axis and defective regulatory mechanisms of the stress response.

Though it was anticipated that decreased CR numbers would be observed in our animal model studies, the possibility cannot be excluded also that mechanisms operative in provoking alterations in various models are different. Therefore generalisations concerning the impact of stressors on different parameters should be approached with caution. Different environmental stressors are likely to trigger different neurotransmitter pathways and neuroendocrine systems therefore resulting in a different underlying neurobiological state of the organism. That depression may be associated with other clinical diagnoses such as anxiety has been long recognised. Thus, anxiety, stress and depression are inter-related and it is possible that the depressive syndrome may be the cumulative effect of chronic exposure to stress and anxiety-inducing stimuli. A multivariate approach is needed to adequately assess the specificity of effects of any given manipulations.

Contemporary therapy of major depression is dominated by drug therapy, this dependence being unlikely to change. Even if direct effects of antidepressants on CR are established and lead to modulation of CR as treatment for depression, anti-glucocorticoid agents, though useful, are not likely to become first line option in the treatment of depression. In many clinical trials of anti-glucocorticoid drugs, glucocorticoid supplimentations have to be given with plasma glucocorticoid levels monitored closely and doses being adjusted accordingly (Brown *et al*, 1999; Patten *et al*, 2000). The potential side effects of these drugs may also prove to be inconvenient. Given the enormous interactive capabilities of various stress systems and the unspecific effects of antidepressants after chronic administration, it is possible that drugs developed to target several systems simultaneously (eg. specifically up-regulate GRs and 5-HT_{1A} receptors) may prove to be superior.

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APPENDICES

APPENDIX 2.3.1.

Drugs and chemicals

ITEM	DESCRIPTION	SUPPLIER
Bovine serum albumin	Fraction V	Sigma-Aldrich Co. Ltd, U.K.
Cupric sulphate	$CuSO_4.5H_2O$	AnalaR, U.K.
Dithiothreitol	$C_4H_{10}O_2S_2$	Sigma-Aldrich Co. Ltd, U.K.
1, 2, 4, 6, 7 [^{3}H-dexamethasone]	Concentration = 1mCi/mmol	Amersham Int. Plc, U.K.
Ethylenediamine-tetraacetic acid (EDTA)	$C_{10}H_{14}N_2O_8Na_2.2H_2O$	Sigma-Aldrich Co. Ltd, U.K.
Folin Ciocalteu's Reagant		Sigma-Aldrich Co. Ltd, U.K.
Glycerol (approx. 99%)	$C_3H_8O_3$	Sigma-Aldrich Co. Ltd, U.K.
Hydrocortisone (11β, 17α, 21-trihydroxypregn-4-ene, 3.20-dione)	$C_{21}H_{30}O_5$	Sigma-Aldrich Co. Ltd, U.K.
Optiphase Safe scintillation cocktail		Wallac, U.K.
Polyethylenimine (PEI)	50 wt % solution in water	Sigma-Aldrich Co. Ltd, U.K.
RU28362 (11β, 17α,dihydroxy-6-methyl-17α (prop-1-ynyl)androsta 1, 4, 6 trien-3-one)		Roussel-Uclaf, France.
Scintillation vials		Canberra-Packard Int., U.K.
Sodium carbonate	Anhydrous	BDH, U.K.
Sodium hydroxide	Pellets	ProLabo, U.K.
Sodium molybdate	$NaNO_4.2H_2O$	Sigma-Aldrich Co. Ltd, U.K.
Sucrose	$C_{12}H_{22}O_{11}$ min 99.5%	Sigma-Aldrich Co. Ltd, U.K.
Trisodium citrate	$C_6H_5Na_3O_7$	Sigma-Aldrich Co. Ltd, U.K.
Whatman GF/F filters	Glass fibre filter sheets (45x67cm)	Whatman Int. Ltd., UK
Tris (hydroxymethyl)-aminomethane (TRIS)	$C_4H_{11}NO_3$	ProLabo, U.K.

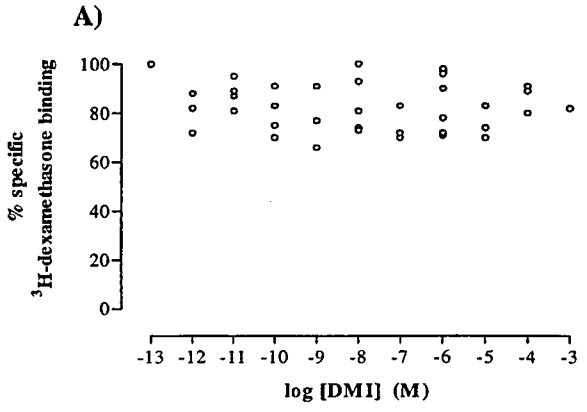
APPENDIX 2.3.1 (continued)

Drugs and Chemicals

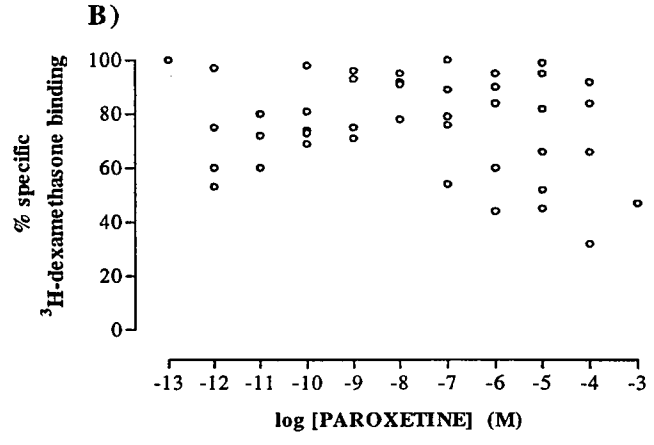
DRUG	SUPPLIER
Citalopram hydrobromide	Lundbeck & Co., Copenhagen, Denmark.
Desipramine hydrochloride	Sigma-Aldrich Co. Ltd, Dorset, U.K.
Paroxetine hydrochloride	SmithKline Beecham, U.K.
Milnacipran hydrochloride	Centre de development, Pierre Fabre, Castres, France.
Venlafaxine hydrochloride	Wyeth-Ayerst Laboratories, Taplow, U.K.

APPENDIX 3.4.1.

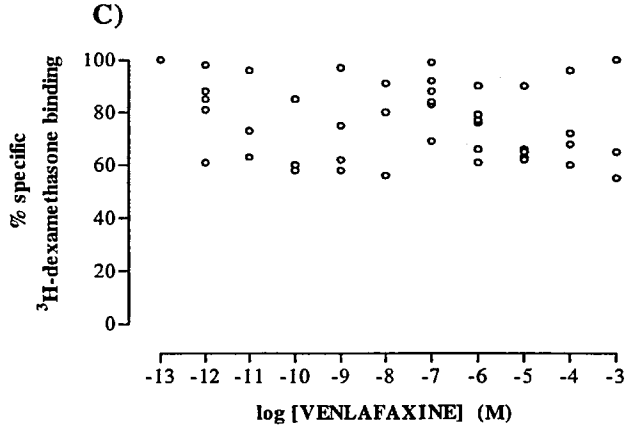
Displacement of ^{3}H-dexamethasone binding from CR in rat cytosolic preparations by DMI (A), paroxetine (B) and venlafaxine (C). Rat cytosolic fractions were prepared as described in section 2.5.2. Data shown represents individual replicates from four experiments.



No displacement of specific ^{3}H-dexamethasone binding to cortical CR by DMI.



No displacement of specific ^{3}H-dexamethasone binding to cortical CR by paroxetine.



No displacement of specific ^{3}H-dexamethasone binding to cortical CR by venlafaxine.

APPENDIX 3.4.2.

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II / II CR/GR** and tissue protein content in **cortex** from animals receiving vehicle or DMI.

(A) see Figure 3.4.2.i

DAYS	VEHICLE					DMI				
	B_{max} CR type I+II	B_{max} CR type II	K_D CR type I+II	K_D CR Type II	protein content	B_{max} CR type I+II	B_{max} CR type II	K_D CR type I+II	K_D CR type II	protein content
1	114 ± 12 (8)	97 ± 14 (8)	3.6 ± 0.4 (8)	2.6 ±0.49 (8)	161 ± 9 (8)	103 ± 10 (8)	102 ± 8 (8)	2.1 ±0.4* (8)	2.3 ±0.4 (8)	187 ± 8 (8)
3	109 ± 7 (8)	94 ± 4 (8)	2.8 ± 0.5 (8)	2.4 ±0.32 (8)	201± 9 (8)	100 ± 4 (7)	85 ± 6 (7)	2.1 ± 0.2 (7)	1.7 ± 0.3 (7)	196 ± 6 (7)
7	83 ± 14 (6)	84 ± 12 (7)	4 ± 1 (6)	4.2 ± 0.7 (7)	171 ± 7 (7)	85 ± 12 (7)	103 ± 7 (7)	3.3 ± 0.5 (7)	3.8 ± 0.5 (7)	183 ± 5 (7)
14	92 ± 6 (7)	85 ± 7 (7)	1.6 ± 0.2 (7)	1.6 ±0.21 (7)	196 ± 7 (7)	125 ±11* (8)	103 ± 7 (8)	1.6 ± 0.2 (8)	1.3 ± 0.2 (8)	207 ± 4 (8)
21	112 ± 5 (8)	105 ± 5 (8)	2.7 ± 0.5 (8)	2.6 ± 0.6 (8)	181 ± 8 (8)	136 ± 11 (8)	129 ± 14 (8)	2.8 ± 0.6 (8)	2.7 ± 0.9 (8)	188 ± 9 (8)
28	121 ± 10 (8)	117 ± 10 (8)	3.3 ± 0.4 (8)	2.7 ±0.21 (8)	179 ± 6 (8)	108 ± 17 (6)	102 ± 16 (6)	2.6 ± 0.4 (6)	2.5 ± 0.4 (6)	173±10 (6)
7 w.d	117 ± 13 (8)	120 ± 15 (8)	1.8 ± 0.6 (8)	2.0 ±0.74 (8)	177 ± 3 (8)	135 ± 16 (8)	136 ± 15 (8)	2.3 ± 0.6 (8)	2.2 ± 0.5 (8)	157±11 (8)

Rats received DMI (10 mg/kg, p.o) or water vehicle once daily for up to 28 days and were sacrificed after 1, 3, 7, 14, 21 and 28 days of administration or 7 days after withdrawal of the drug (7 wd). All procedures and CR assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses =n per group. Individual B_{max} values ranged from 55 - 172 fmol/mg protein in vehicle treated animals and 36 - 214 fmol/mg protein in DMI treated animals. Individual K_D values ranged from 0.34 - 7.94 nM in vehicle treated animals and 0.42 – 6.59 nM in DMI treated animals. Individual protein content values ranged from 132 - 243 µg/assay in vehicle treated animals and 126 - 222 µg/assay in DMI treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$, as compared to respective vehicle treated control group.

APPENDIX 3.4.2. (continued)

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II / II CR/GR** and tissue protein content in **hippocampus** from animals receiving vehicle or DMI.

(B) see Figure 3.4.2.ii

DAYS	VEHICLE					DMI				
	B_{max} CR type I+II	B_{max} CR type II	K_D CR type I+II	K_D CR Type II	protein content	B_{max} CR type I+II	B_{max} CR type II	K_D CR type I+II	K_D CR type II	protein content
1	118 ± 10 (8)	114 ± 9 (8)	2.1 ± 0.5 (8)	2 ± 0.51 (8)	131±11 (8)	119 ± 8 (7)	110 ± 8 (7)	3.3 ± 1.0 (7)	3.1 ± 0.9 (7)	145±12 (7)
3	79 ± 11 (8)	75 ± 22 (8)	4.3 ± 1 (8)	3.4 ± 1.1 (8)	146 ± 9 (8)	64 ± 8 (7)	62 ± 9 (7)	2.7 ± 0.6 (7)	2.7 ± 0.6 (7)	138 ± 8 (7)
7	99 ± 9 (7)	95 ± 9 (7)	4.3 ± 1 (7)	4.7 ± 1.5 (7)	146±13 (7)	112 ± 10 (7)	105 ± 12 (7)	4.1 ± 0.8 (7)	3.9 ± 0.8 (7)	130 ± 9 (7)
14	95 ± 13 (8)	82 ± 11 (8)	2.5 ± 0.6 (8)	1.9 ± 0.2 (8)	143 ± 8 (8)	102 ± 6 (8)	84 ± 9 (8)	1.9 ± 0.4 (8)	1.1 ± 0.1 (8)	146 ± 8 (8)
21	99 ± 12 (8)	100 ± 8 (8)	2.3 ± 0.5 (8)	2.4 ± 0.5 (8)	171 ± 7 (8)	97 ± 10 (8)	94 ± 9 (8)	1.6 ± 0.3 (8)	1.6 ± 0.2 (8)	157±14 (8)
28	96 ± 11 (7)	102 ± 11 (7)	2.7 ± 0.5 (7)	3.1 ± 0.8 (7)	124 ± 9 (7)	94 ± 11 (7)	86 ± 8 (7)	3.0 ± 0.2 (7)	3 ± 0.8 (7)	136 ± 8 (7)
7 w.d	108 ± 9 (7)	106 ± 7 (7)	1.1 ± 0.2 (7)	1.2 ± 0.3 (7)	147 ± 9 (7)	110 ± 6 (8)	108 ± 7 (8)	1.4 ± 0.5 (8)	1.3 ± 0.3 (8)	137 ± 8 (8)

Rats received DMI (10 mg/kg, p.o) or water vehicle once daily for up to 28 days and were sacrificed after 1, 3, 7, 14, 21 and 28 days of administration or 7 days after withdrawal of the drug (7 wd). All procedures and CR assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses =n per group. Individual B_{max} values ranged from 50 - 180 fmol/mg protein in vehicle treated animals and 33 - 155 fmol/mg protein in DMI treated animals. Individual K_D values ranged from 0.38 - 7.86 nM in vehicle treated animals and 0.42- 7.57 nM in DMI treated animals. Individual protein content values ranged from 79 - 210 µg/assay in vehicle treated animals and 91 - 221 µg/assay in DMI treated animals. No statistically significant differences were determined using ANOVA followed by Student's t-tests.

APPENDIX 3.4.3.

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I+II CR** and tissue protein content in **cortex** (A) taken from animals receiving vehicle or paroxetine.

(A) See Figure 3.4.3.i

DAYS	VEHICLE			PAROXETINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	101 ± 18 (8)	2.7 ± 0.5 (8)	145 ± 7 (8)	115 ± 13 (8)	1.9 ± 0.4 (8)	173 ± 7 (8)
7	93 ± 11 (7)	2.5 ± 0.7 (7)	160 ± 5 (7)	104 ± 9 (7)	2.3 ± 0.5 (7)	150 ± 8 (7)
14	114 ± 13 (7)	2.7 ± 0.7 (7)	157 ± 8 (7)	76 ± 3** (8)	2.3 ± 0.6 (7)	159 ± 6 (8)
28	102 ± 9 (8)	2.5 ± 0.6 (8)	153 ± 7 (8)	92 ± 8 (7)	2.8 ± 0.7 (7)	167 ± 6 (7)
7 w.d	103 ± 12 (8)	2.3 ± 0.8 (8)	157 ± 4 (8)	101 ± 12 (7)	2.1 ± 0.5 (7)	165 ± 7 (7)

Rats received paroxetine (5 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1, 7, 14 and 28 days of administration or 7 days after withdrawal of the drug (7 w.d). All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group. Individual B_{max} values ranged from 44 - 193 fmol/mg protein in vehicle treated animals and 39 - 154 fmol/mg protein in paroxetine treated animals. Individual K_D values ranged from 0.73 – 7.32 nM in vehicle treated animals and 0.81 – 5.34 nM in paroxetine treated animals. Individual protein content values ranged from 119 - 178 µg/assay in vehicle treated animals and 99 - 214 µg/assay in paroxetine treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by ** $p<0.01$, as compared to respective vehicle treated control groups.

APPENDIX 3.4.3. (continued)

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** and tissue protein content in **hippocampus** (B) taken from animals receiving vehicle or paroxetine.

(B) see Figure 3.4.3.ii

DAYS	VEHICLE			PAROXETINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	69 ± 13 (7)	3.4 ± 0.6 (7)	164 ± 6 (7)	75 ± 10 (7)	3.8 ± 0.6 (7)	156 ± 6 (7)
7	59 ± 9 (7)	2.4 ± 0.6 (7)	147 ± 4 (7)	79 ± 13 (8)	3.2 ± 0.9 (8)	146 ± 6 (8)
14	67 ± 11 (8)	2.6 ± 0.9 (8)	156 ± 5 (8)	71 ± 12 (8)	3.1 ± 0.7 (8)	142 ± 7 (8)
28	99 ± 15 (6)	5.2 ± 0.7 (6)	150 ± 7 (8)	73 ± 7 (8)	3 ± 0.6* (8)	147 ± 5 (8)
7 w.d	73 ± 14 (8)	3.3 ± 0.9 (8)	160 ± 5 (8)	94 ± 18 (7)	3.5 ± 0.8 (7)	156 ± 6 (7)

Rats received paroxetine (5 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1, 7, 14 and 28 days of administration or 7 days after withdrawal of the drug (7 w.d). All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group. Individual B_{max} values ranged from 21 - 154 fmol/mg protein in vehicle treated animals and 39 - 185 fmol/mg protein in paroxetine treated animals. Individual K_D values ranged from 0.59 - 8 nM in vehicle treated animals and 0.89 – 7.39 nM in paroxetine treated animals. Individual protein content values ranged from 127 - 192 µg/assay in vehicle treated animals and 108 - 177 µg/assay in paroxetine treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$ as compared to respective vehicle treated control groups.

APPENDIX 3.4.3. (continued)

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** and tissue protein content in **striatum** (C) taken from animals receiving vehicle or paroxetine.

(C) see Figure 3.4.3.iii

DAYS	VEHICLE			PAROXETINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	95 ± 17 (3)	4.5 ± 0.8 (3)	137 ± 7 (3)	83 ± 3 (3)	3.7 ± 1 (3)	141 ± 5 (3)
7	66 ± 6 (4)	3.1 ± 1.3 (4)	143 ± 3 (4)	88 ± 4* (4)	5.5 ± 1.2 (4)	143 ± 10 (4)
14	79 ± 11 (4)	3 ± 0.48 (4)	148 ± 5 (4)	98 ± 15 (3)	4.1 ± 1 (3)	132 ± 6 (4)
28	107 ± 27 (4)	4.1 ± 1 (4)	135 ± 2 (4)	76 ± 11 (4)	3 ± 0.7 (4)	153 ± 7* (4)
7 w.d	88 ± 9 (4)	3.2 ± 0.9 (4)	145 ± 5 (4)	61 ± 9 (4)	1.8 ± 0.4 (4)	133 ± 16 (4)

Rats received paroxetine (5 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1, 7, 14 and 28 days of administration or 7 days after withdrawal of the drug (7 w.d). All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group (tissues were pooled from two animals in these experiments). Individual B_{max} values ranged from 52 - 185 fmol/mg protein in vehicle treated animals and 36 - 114 fmol/mg protein in paroxetine treated animals. Individual K_D values ranged from 1.12 - 6.89 nM in vehicle treated animals and 1.02 – 6.99 nM in paroxetine treated animals. Individual protein content values ranged from 123 - 157 µg/assay in vehicle treated animals and 121 - 170 µg/assay in paroxetine treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$, as compared to respective vehicle treated control groups.

APPENDIX 3.4.3. (continued)

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** and tissue protein content in **thymus** (D) taken from animals receiving vehicle or paroxetine.

(D) see Figure 3.4.3.iv

DAYS	VEHICLE			PAROXETINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	417 ± 30 (8)	2.8 ± 0.7 (8)	156 ± 7 (8)	433 ± 34 (7)	2.5 ± 0.6 (7)	152±10 (7)
28	336 ± 22 (8)	3.3 ± 0.3 (8)	160 ± 5 (8)	257 ± 22* (8)	2.7 ± 0.3 (8)	163±10 (8)

Rats received paroxetine (5 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1 and 28 days of administration. All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group. Individual B_{max} values ranged from 227 - 601 fmol/mg protein in vehicle treated animals and 155 - 615 fmol/mg protein in paroxetine treated animals. Individual K_D values ranged from 1.28 – 6.93 nM in vehicle treated animals and 1.34 – 5.6 nM in paroxetine treated animals. Individual protein content values ranged from 127 - 168 µg/assay in vehicle treated animals and 135 - 176 µg/assay in paroxetine treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$, as compared to respective vehicle treated control groups.

APPENDIX 3.4.4.

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** and tissue protein content in **cortex** (A) taken from animals receiving vehicle or venlafaxine.

(A) see Figure 3.4.4.i

DAYS	VEHICLE			VENLAFAXINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	121 ± 17 (8)	3.1 ± 0.7 (8)	157 ± 5 (8)	120 ± 14 (8)	2.4 ± 0.6 (8)	146 ± 5 (8)
7	123 ± 16 (8)	3.2 ± 1 (8)	147 ± 5 (8)	127 ± 15 (8)	2.3 ± 0.3 (8)	142 ± 4 (8)
14	116 ± 10 (8)	2.2 ± 0.5 (8)	140 ± 8 (8)	126 ± 11 (8)	4.7 ± 1* (8)	158 ± 8 (8)
28	121 ± 13 (7)	2.2 ± 0.6 (7)	148 ± 3 (7)	149 ± 7 (8)	3.8 ± 0.7 (8)	151 ± 3 (8)
7 w.d	119 ± 11 (8)	3.1 ± 0.8 (8)	144 ± 7 (8)	129 ± 11 (8)	2.7 ± 0.5 (8)	152 ± 6 (8)

Rats received venlafaxine (15 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1, 7, 14 and 28 days of administration or 7 days after withdrawal of the drug (7 w.d). All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group. Individual B_{max} values ranged from 58 - 223 fmol/mg protein in vehicle treated animals and 38 - 198 fmol/mg protein in venlafaxine treated animals. Individual K_D values ranged from 0.23 – 7.59 nM in vehicle treated animals and 0.52 – 6.8 nM in venlafaxine treated animals. Individual protein content values ranged from 107 - 185 µg/assay in vehicle treated animals and 119 - 204 µg/assay in venlafaxine treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$, as compared to respective vehicle treated control groups.

APPENDIX 3.4.4. (continued)

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** and tissue protein content in **hippocampus** (B) taken from animals receiving vehicle or venlafaxine.

(B) see Figure 3.4.4.ii

DAYS	VEHICLE			VENLAFAXINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	109 ± 14 (8)	2.1 ± 0.3 (8)	108 ± 9 (8)	127 ± 20 (7)	3.5 ± 0.8 (7)	108 ± 8 (7)
7	116 ± 12 (8)	2.6 ± 0.6 (8)	116 ± 4 (8)	129 ± 16 (7)	2.8 ± 0.4 (7)	118 ± 4 (7)
14	106 ± 11 (8)	2.3 ± 0.4 (8)	108 ± 7 (8)	118 ± 8 (8)	4.2±0.7* (8)	115 ± 7 (8)
28	112 ± 7 (8)	2.4 ± 0.5 (8)	119 ± 3 (8)	139 ± 12 (8)	3.2 ± 0.5 (8)	114 ± 6 (8)
7 w.d	104 ± 5 (8)	2 ± 0.31 (8)	123 ± 7 (8)	102 ± 10 (8)	1.5 ± 0.3 (8)	119 ± 4 (8)

Rats received venlafaxine (15 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1, 7, 14 and 28 days of administration or 7 days after withdrawal of the drug (7 w.d). All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group. Individual B_{max} values ranged from 39 - 174 fmol/mg protein in vehicle treated animals and 38 - 208 fmol/mg protein in venlafaxine treated animals. Individual K_D values ranged from 0.7 – 5.94 nM in vehicle treated animals and 0.4 – 7.17 nM in venlafaxine treated animals. Individual protein content values ranged from 62 - 160 µg/assay in vehicle treated animals and 84 - 150 µg/assay in venlafaxine treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$, as compared to respective vehicle treated control groups.

APPENDIX 3.4.4. (continued)

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** and tissue protein content in **striatum** (C) taken from animals receiving vehicle or venlafaxine.

(C) see Figure 3.4.4.iii

DAYS	VEHICLE			VENLAFAXINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	112 ± 5 (4)	2.5 ± 0.8 (4)	129 ± 4 (4)	134 ± 9 (4)	2 ± 0.5 (4)	132 ± 7 (4)
7	167 ± 22 (4)	2.7 ± 1 (4)	136 ± 6 (4)	152 ± 7 (3)	1.8 ± 0.5 (3)	138 ± 1 (4)
14	122 ± 31 (4)	1.8 ± 0.8 (4)	127 ± 7 (4)	105 ± 26 (4)	1.7 ± 0.8 (4)	130±11 (4)
28	123 ± 17 (4)	1.8 ± 0.7 (4)	146 ± 7 (4)	191 ± 56 (4)	2.8 ± 1.9 (4)	145±11 (4)
7 w.d	170 ± 28 (4)	3.3 ± 0.4 (4)	122 ± 8 (4)	121 ± 23 (4)	1.5 ± 0.3 * (4)	141 ± 4 (4)

Rats received venlafaxine (15 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1, 7, 14 and 28 days of administration or 7 days after withdrawal of the drug (7 w.d). All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group (tissues were pooled from two animals in these experiments). Individual B_{max} values ranged from 66 - 227 fmol/mg protein in vehicle treated animals and 44 – 336 fmol/mg protein in venlafaxine treated animals. Individual K_D values ranged from 0.51 – 5.17 nM in vehicle treated animals and 0.56 – 6.58 nM in venlafaxine treated animals. Individual protein content values ranged from 99 - 164 µg/assay in vehicle treated animals and 101 - 156 µg/assay in venlafaxine treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$, as compared to respective vehicle treated control groups.

APPENDIX 3.4.4. (continued)

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** and tissue protein content in **thymus** (D) taken from animals receiving vehicle or venlafaxine.

(D) see Figure 3.4.4.iv

DAYS	VEHICLE			VENLAFAXINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	278 ± 47 (8)	4.3 ± 1 (8)	111 ± 4 (8)	245 ± 54 (8)	3.3 ± 0.6 (8)	109 ± 3 (8)
14	220 ± 28 (8)	4.2 ± 0.9 (8)	115 ± 3 (8)	219 ± 27 (8)	2.6 ± 0.6 (8)	111 ± 4 (8)
28	262 ± 48 (8)	4.2 ± 0.7 (8)	116 ± 5 (8)	302 ± 54 (7)	4.7 ± 0.7 (7)	102 ± 9 (7)

Rats received venlafaxine (15 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1, 14 and 28 days of administration. All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group. Individual B_{max} values ranged from 74 - 519 fmol/mg protein in vehicle treated animals and 106 - 532 fmol/mg protein in venlafaxine treated animals. Individual K_D values ranged from 1.4 – 8.65 nM in vehicle treated animals and 1.06 – 7.2 nM in venlafaxine treated animals. Individual protein content values ranged from 93 - 133 µg/assay in vehicle treated animals and 64 - 126 µg/assay in venlafaxine treated animals. No statistically significant differences were determined using ANOVA followed by Student's t-tests.

APPENDIX 3.4.4. (continued)

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** and tissue protein content in **hypothalamus** (E) taken from animals receiving vehicle or venlafaxine.

(E) see Figure 3.4.4.v

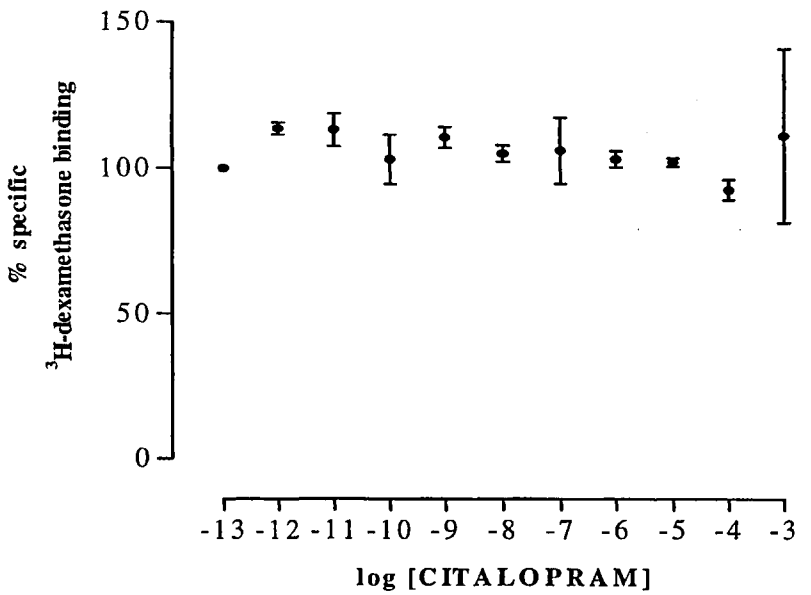
Time	VEHICLE			VENLAFAXINE		
	B_{max} CR type I+II	K_D CR type I+II	protein content	B_{max} CR type I+II	K_D CR type I+II	protein content
1	121 ± 6 (4)	2.23 ± 0.51 (4)	102 ± 11 (4)	133 ± 13 (4)	3.17 ± 1.35 (4)	125 ± 21 (4)
7	142 ± 16 (4)	2.61 ± 0.75 (4)	140 ± 4 (4)	130 ± 17 (4)	2.63 ± 0.6 (4)	115 ± 13 (4)
14	129 ± 29 (4)	3.44 ± 0.65 (4)	103 ± 13 (4)	104 ± 21 (4)	2.37 ± 0.66 (4)	125 ± 10 (4)
28	139 ± 22 (4)	2.16 ± 0.33 (4)	143 ± 11 (4)	174 ± 23 (4)	3.85 ± 0.84 (4)	120 ± 15 (4)
7 w.d	88 ± 8 (4)	1.59 ± 0.71 (4)	115 ± 14 (4)	150 ± 31 (4)	2.51 ± 0.69 (4)	144 ± 9 * (4)

Rats received venlafaxine (15 mg/kg, p.o.) or distilled water vehicle once daily for up to 28 days and were sacrificed after 1, 7, 14 and 28 days of administration or 7 days after withdrawal of the drug (7 w.d). All procedures and CR binding assays were conducted as described in section 3.2. Data are expressed as mean ± sem. Numbers in parentheses = n per group (tissues were pooled from two animals in these experiments). Individual B_{max} values ranged from 62 - 200 fmol/mg protein in vehicle treated animals and 61 -243 fmol/mg protein in venlafaxine treated animals. Individual K_D values ranged from 0.48 – 4.69 nM in vehicle treated animals and 0.87 – 5.21 nM in venlafaxine treated animals. Individual protein content values ranged from 77 - 172 µg/assay in vehicle treated animals and 67 - 166 µg/assay in venlafaxine treated animals. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$, as compared to respective vehicle treated control groups.

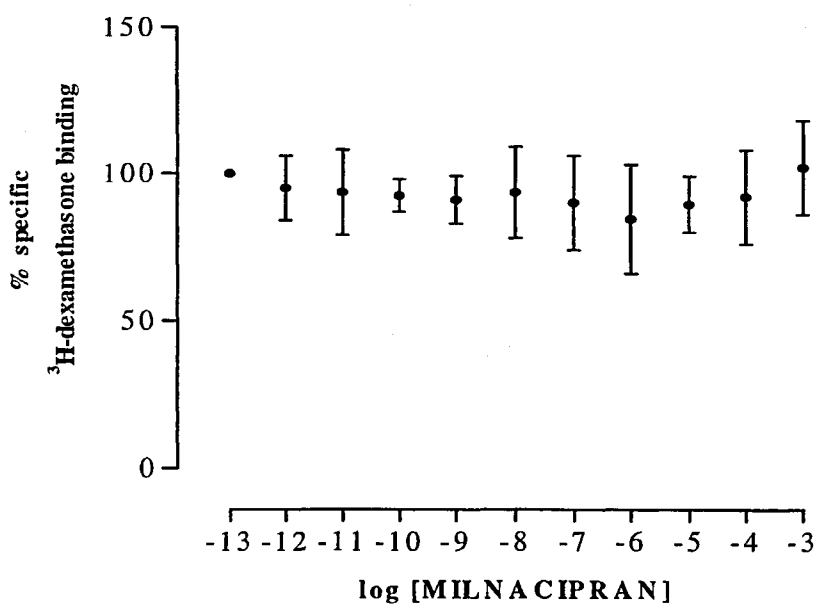
APPENDIX 4.3.1.

Displacement of ^{3}H-dexamethasone binding from CR in rat cytosolic preparations by **citalopram (A) & milnacipran (B).** Rat cytosolic fractions were prepared as described in section 2.5.2. Data shown is taken from a single experiment.

(A)



(B)



APPENDIX 4.3.3.

The effects of acute and chronic administration of **citalopram (A), venlafaxine (B), DMI (C) and milnacipran (D)** on grooming scores in the open field of sham-operated (SO) and olfactory bulbectomised (OB) rats (Data not displayed in Chapter 4).

(A)

	SO + vehicle	**SO + citalopram**	**OB + vehicle**	**OB + citalopram**
DAY 3				
grooming	0.6 ± 0.3	0.2 ± 0.2	0.1 ± 0.1	0.6 ± 0.4
DAY 28				
grooming	0.0 ± 0.0	0.1 ± 0.1	0.0 ± 0.0	0.0 ± 0.0

(B)

	SO + vehicle	**SO + venlafaxine**	**OB + vehicle**	**OB + venlafaxine**
DAY 3				
grooming	0.6 ± 0.3	0.3 ± 0.3	0.1 ± 0.1	0.3 ± 0.3
DAY 28				
grooming	0.0 ± 0.0	0.3 ± 0.3	0.0 ± 0.0	0.0 ± 0.0

(C)

	SO + vehicle	**SO + DMI**	**OB + vehicle**	**OB + DMI**
DAY 3				
grooming	0.6 ± 0.3	0.9 ± 0.6	0.1 ± 0.1	0.0 ± 0.0
DAY 28				
grooming	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

APPENDIX 4.3.3. (continued)

(D)

	SO + vehicle	SO + milnacipran	OB + vehicle	OB + milnacipran
DAY 3				
grooming	0.6 ± 0.3	0.1 ± 0.1	0.1 ± 0.1	0.5 ± 0.3
DAY 28				
grooming	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0

Rats (7-10 per group) received citalopram (5mg/kg p.o.) or distilled water twice daily and were tested for open-field activity approximately 16 hours following the last dose as described in section 4.2.4. Data are expressed as mean ± sem. Statistically significant differences are not displayed as the data were too erratic and standard errors too large for statistical determinations (used ANOVA followed by Students t-tests where appropriate (GB-STAT v6.0)).

APPENDIX 4.3.4.i

Grooming (A) and **defecation (B)** scores in sham-operated (SO) rats following administration of vehicle, citalopram, venlafaxine, DMI or milnacipran for 3, 7, 10, 14, 21 and 28 days (data not displayed in Chapter 4).

(A) grooming

	SO + vehicle	SO + citalopram	SO + venlafaxine	SO + DMI	SO + milnacipran
Day 3	0.6 ± 0.3	0.2 ± 0.2	0.2 ± 0.3	0.9 ± 0.6	0.1 ± 0.1
Day 7	0.6 ± 0.3	0.4 ± 0.2	0.6 ± 0.3	0.9 ± 0.3	0.6 ± 0.4
Day 10	0.1 ± 0.1	0.3 ± 0.2	0.0 ± 0.0	0.1 ± 0.1	0.1 ± 0.1
Day 14	0.0 ± 0.0	0.2 ± 0.2	1.1 ± 0.4	0.4 ± 0.2	0.6 ± 0.4
Day 21	0.3 ± 0.2	0.1 ± 0.1	0.4 ± 0.4	0.4 ± 0.4	0.0 ± 0.0
Day 28	0.0 ± 0.0	0.1 ± 0.1	0.3 ± 0.3	0.0 ± 0.0	0.1 ± 0.1

(B) defecation

	SO + vehicle	SO + citalopram	SO + venlafaxine	SO + DMI	SO + milnacipran
Day 3	3.6 ± 0.6	3.1 ± 0.7	3.6 ± 1.3	4.8 ± 1.0	2.3 ± 0.7
Day 7	3.6 ± 0.8	3.0 ± 0.9	3.6 ± 1.1	3.9 ± 0.6	3.4 ± 0.8
Day 10	2.1 ± 0.5	3.6 ± 0.9	1.3 ± 0.6	2.2 ± 0.7	1.6 ± 0.9
Day 14	1.6 ± 0.9	3.8 ± 1.0	3.5 ± 0.8	3.2 ± 1.1	3.0 ± 0.9
Day 21	4.6 ± 1.0	3.6 ± 0.7	2.4 ± 1.0	5.1 ± 0.9	4.3 ± 0.9
Day 28	4.3 ± 0.8	3.4 ± 0.7	2.5 ± 0.7	3.5 ± 0.8	2.3 ± 1.0

Rats (7-10 per group) received citalopram, venlafaxine, DMI or milnacipran twice daily over 28 days and wer tested for open field activity approximately 16 hours after the last dose as described in section 4.2.4. Data are expressed as mean ± sem. Statistically significant differences were not determined for grooming scores as these were too erratic with very large sem values. Statistically significant differences for defecation values were determined using ANOVA followed by Student's t-tests where appropriate and are denoted by * $p<0.05$. ***ANOVA: Citalopram*** *- effect of treatment $F(1, 84) = 0.95$, $p=0.33$: effect of time $F(5, 84) = 0.43$, $p=0.83$; interaction treatment x time $F(5, 84) - 1.86$, $p=0.11$..* ***Venlafaxine*** *- effect of treatment $F(1, 76) = 2.87$, $p=0.11$: effect of time $F(5, 76) = 1.56$, $p=0.18$; interaction treatment x time $F(5, 76) - 1.02$, $p=0.41$.* ***DMI*** *- effect of treatment $F(1, 84) = 4.53$, $p=0.37$: effect of time $F(5, 84) = 1.78$, $p=0.13$; interaction treatment x time $F(5, 84) - 1.45$, $p=0.22$..* ***Milnacipran*** *- effect of treatment $F(1, 75) = 1.87$, $p=0.49$: effect of time $F(5, 75) = 0.42$, $p=0.83$; interaction treatment x time $F(5, 75) - 1.36$, $p=0.25$.*

APPENDIX 4.3.4.ii.

^{3}H-dexamethasone binding parameters (B_{max} and K_D) to **type I + II CR** in hippocampus taken from sham-operated (SO) animals receiving **citalopram (A), venlafaxine (B), DMI (C)** or **milnacipran (D)** over 28 days.

(A) see Figure 4.3.4.i

DAYS	VEHICLE		CITALOPRAM	
	B_{max} CR (fmol/mg tissue)	K_D CR (nM)	B_{max} CR (fmol/mg tissue)	K_D CR (nM)
3	2.78 ± 0.3 (7)	0.95 ± 0.12 (7)	3.1 ± 0.12 (7)	1.03 ± 0.12 (7)
7	2.97 ± 0.29 (8)	1.19 ± 0.15 (8)	3.92 ± 0.51 (8)	1.28 ± 0.17 (8)
10	3.01 ± 0.24 (8)	2.27 ± 0.45 (8)	3.56 ± 0.21 (8)	1.96 ± 0.3 (8)
21	2.67 ± 0.16 (8)	1.77 ± 0.35 (8)	2.62 ± 0.16 (8)	1.07 ± 0.1 (8)
21	2.29 ± 0.26 (8)	1.22 ± 0.31 (8)	2.16 ± 0.17 (8)	0.97 ± 0.1 (8)
28	2.53 ± 0.28 (8)	1.32 ± 0.3 (8)	3.2 ± 0.19 (8)	1.47 ± 0.21 (8)

(B) see Figure 4.3.4.ii

DAYS	VEHICLE		VENLAFAXINE	
	B_{max} CR (fmol/mg tissue)	K_D CR (nM)	B_{max} CR (fmol/mg tissue)	K_D CR (nM)
3	2.78 ± 0.3 (7)	0.95 ± 0.12 (7)	2.93 ± 0.28 (8)	1.32 ± 0.3 (8)
7	2.97 ± 0.29 (8)	1.19 ± 0.15 (8)	4.0 ± 0.62 (8)	1.84 ± 0.40 (8)
10	3.01 ± 0.24 (8)	2.27 ± 0.45 (8)	3.12 ± 0.39 (8)	1.69 ± 0.42 (8)
21	2.67 ± 0.16 (8)	1.77 ± 0.35 (8)	2.54 ± 0.09 (8)	1.11 ± 0.11 (8)
21	2.29 ± 0.26 (8)	1.22 ± 0.31 (8)	2.28 ± 0.25 (8)	1.21 ± 0.18 (8)
28	2.53 ± 0.28 (8)	1.32 ± 0.3 (8)	2.59 ± 0.2 * (8)	2.11 ± 0.11 * (8)

(C) see Figure 4.3.4.iii

DAYS	VEHICLE		DMI	
	B_{max} CR (fmol/mg tissue)	K_D CR (nM)	B_{max} CR (fmol/mg tissue)	K_D CR (nM)
3	2.78 ± 0.3 (7)	0.95 ± 0.12 (7)	3.65 ± 0.40 (8)	0.92 ± 0.06 (8)
7	2.97 ± 0.29 (8)	1.19 ± 0.15 (8)	5.17 ± 1.25 (6)	1.22 ± 0.22 (6)
10	3.01 ± 0.24 (8)	2.27 ± 0.45 (8)	3.78 ± 0.37 (8)	1.42 ± 0.27 (8)
21	2.67 ± 0.16 (8)	1.77 ± 0.35 (8)	2.69 ± 0.12 (8)	1.26 ± 0.09 (8)
21	2.29 ± 0.26 (8)	1.22 ± 0.31 (8)	2.62 ± 0.20 (8)	0.93 ± 0.13 (8)
28	2.53 ± 0.28 (8)	1.32 ± 0.3 (8)	3.13 ± 0.29 (8)	1.71 ± 0.38 (8)

(D) see Figure 4.3.4.iv

DAYS	VEHICLE		MILNACIPRAN	
	B_{max} CR (fmol/mg tissue)	K_D CR (nM)	B_{max} CR (fmol/mg tissue)	K_D CR (nM)
3	2.78 ± 0.3 (7)	0.95 ± 0.12 (7)	2.94 ± 0.25 (8)	0.95 ± 0.12 (8)
28	2.53 ± 0.28 (8)	1.32 ± 0.3 (8)	3.1 ± 0.31 (8)	1.07 ± 0.36 (8)

Rats received citalopram (5 mg/kg, p.o.), venlafaxine (15 mg/kg, p.o.), DMI (5 mg/kg, p.o.), milnacipran (15 mg/kg, p.o.) or distilled water vehicle twice daily for up to 28 days and were sacrificed after 3, 7, 10, 14, 21 and 28 days of administration. All procedures and CR binding assays were conducted as described in section 3.2. Also see section 4.3.4. Data are expressed as mean ± sem. Numbers in parentheses = n per group. Statistically significant differences were determined using ANOVA followed by Student's t-tests and are denoted by * $p<0.05$ as compared to vehicle treated control groups.

APPENDIX 6.2.3.A.

Open field activity of BALB/c mice following acute exposure to predator stress (see Figure 6.2.3.i).

	IMMEDIATELY AFTER POS		24 HOURS AFTER POS	
	NON-STRESSED	STRESS	NON-STRESSED	STRESS
AMBULATION	20 ± 5	39 ± 8	52 ± 13	24 ± 6
GROOMING (not shown in chap 6)	0.5 ± 0.27	0.88 ± 0.35	0.63 ± 0.18	0.63 ± 0.26
REARING (not shown in chap 6)	0.25 ± 0.16	0.13 ± 0.13	0.25 ± 1.63	1.38 ± 0.96
DEFECATION	2.88 ± 0.72	1.13 ± 0.48	4 ± 4.6	4.13 ± 0.97
MOBILITY LATENCY	160 ± 35	14 ± 4 **	37 ± 11	94 ± 37

BALB/c mice (n=8 per group) were subjected to open field tests immediately, or 24 hours after exposure to predator stress. Data are expressed as mean ± sem. Table shows scores for ambulation, grooming (not shown in Chapter 6), rearing (data not shown in Chapter 6), defecation and mobility latency immediately and 24 hours after predator exposure. Statistically significant differences were determined using t-tests (ambulation, defecation and mobility latency) or Mann-Whitney U tests (grooming and rearing) and are denoted by ** $p<0.01$.

APPENDIX 6.2.3.B.

Sucrose intake of BALB/c mice following acute exposure to predator stress (see Figure 6.2.3.ii).

(A) IMMEDIATELY AFTER POS		(B) 24 HOURS AFTER POS	
NON-STRESSED	STRESS	NON-STRESSED	STRESS
1.28 ± 0.12	0.74 ± 0.1 **	**0.91 ± 0.06**	1.1 ± 0.15

BALB/c mice (n=10 per group) were subjected to 1-bottle sucrose consumption tests immediately or 24 hours after exposure to predator stress (see section 6.2.3.ii). Data are expressed as mean ± sem in g. **(A)** sucrose intake immediately following exposure to predator stress, **(B)** sucrose intake 24 hours following exposure to predator stress. Statistically significant differences were determined using t-tests (using GB-STAT v6.5) and are denoted by ** $p<0.01$.

APPENDIX 6.2.3.C.

Plasma corticosterone concentrations in BALB/c mice following acute exposure to predator stress (see Figure 6.2.3.iii).

(A) IMMEDIATELY AFTER POS		(B) 24 HOURS AFTER POS	
NON-STRESSED	STRESS	NON-STRESSED	STRESS
28 ± 6	43 ± 6	37 ± 6	26 ± 4

Plasma corticosterone concentrations in BALB/c mice (n=6-7 per group) were assessed in blood samples taken following exposure to predator stress (see section 6.3.2.iii). Data are expressed as mean ± sem (in ng/mL). (A) plasma corticosterone concentrations immediately following exposure to predator stress, (B) plasma corticosterone concentrations 24 hours following exposure to predator stress. No statistically significant differences were determined between control and stressed groups.

APPENDIX 6.2.3.D.

Specific ^{3}H-dexamethasone binding to cortical/hippocampal cytosolic fractions following acute exposure to predator stress (see Figure 6.2.3.iv).

Cortex

	(A) IMMEDIATELY AFTER POS		(B) 24 HOURS AFTER POS	
	NON-STRESSED	STRESS	NON-STRESSED	STRESS
B_{max} (fmol/mg protein)	143 ± 14	147 ± 10	153 ± 6	180 ± 16
K_D	1.02 ± 0.29	1.51 ± 0.54	1.58 ± 0.68	1.67 ± 0.51

Hippocampus (B_{max} only)

(C) IMMEDIATELY AFTER POS		(D) 24 HOURS AFTER POS	
NON-STRESSED	STRESS	NON-STRESSED	STRESS
138 ± 12	131 ± 18	142 ± 3	153 ± 17

Specific ^{3}H-dexamethasone binding to CR in cortex/hippocampus (from BALB/c mice (n=8 per group)) was measured following acute exposure to predator stress. See sections 2.3 and 6.2.3.iv. Data are expressed as mean ± sem (in fmol/mg protein – B_{max} and nM – K_D). (A) B_{max} of cortical CR binding immediately after predator exposure, (B) B_{max} of cortical CR binding 24 hours after predator exposure, (C) K_D of cortical CR binding immediately following predator exposure, (D) K_D of cortical CR binding 24 hours after predator exposure, (E) B_{max} of hippocampal CR binding immediately after predator exposure, (F) B_{max} of hippocampal CR binding 24 hours after predator exposure. No statistically significant differences were found between control and stressed groups.

APPENDIX 6.3.3.A.

Body weights (g) of BALB/c mice following chronic exposure to predator stress and antidepressant administration (see Figures 6.3.3.i.1/2).

WEEK No.	NON-STRESSED CONTROL			STRESSED		
	VEHICLE	DMI	PAROX	VEHICLE	DMI	PAROX
1 (n= 60)	31.1 ± 0.3					
2 (n= 60)	31.1 ± 0.4					
3 (n= 60)	31.4 ± 0.4					
4 (n= 30)	32.4 ± 0.7	32.4 ± 0.7	32.4 ± 0.7	31.4 ± 0.5	31.4 ± 0.5	31.4 ± 0.5
5 (n= 30)	32.6 ± 0.7	32.6 ± 0.7	32.6 ± 0.7	31 ± 0.5	31 ± 0.5	31 ± 0.5
6 (n= 30)	32.8 ± 0.8	32.8 ± 0.8	32.8 ± 0.8	31.8 ± 0.5	31.8 ± 0.5	31.8 ± 0.5
7 (n= 30)	33.7 ± 0.7	33.7 ± 0.7	33.7 ± 0.7	32.7 ± 0.5	32.7 ± 0.5	32.7 ± 0.5
8 (n= 8)	34.1 ± 1.4	36.2 ± 1.02	31.8 ± 1.2	32 ± 0.9	34.9 ± 1.1	32.1 ± 0.8
9 (n= 8)	32.1 ± 1.1	32.6 ± 0.9	29.8 ± 0.9	29.5 ± 0.7 *	29.9 ± 1.4	28.9 ± 0.9
10 (n= 8)	32.5 ± 1.0	33.5 ± 0.74	31.4 ± 1.1	28.7 ± 0.7 *	31.3 ± 1.1	31.1 ± 0.8
11 (n= 8)	32.8 ± 0.9	33.7 ± 0.9	33 ± 1.4	29.6 ± 0.7 *	31.4 ± 1.0	31.9 ± 0.9
12 (n= 8)	33.1 ± 0.9	32.1 ± 1.01	33.8 ± 1.6	29.9 ± 0.7 *	31 ± 1.1	32.7 ± 0.9

Body weights of individually housed mice were recorded before and after the introduction of a predator stress stimulus, see section 6.3.3.i. Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5 mg/kg) began at the start of week 8 for 28 days. Data are expressed as mean ± sem (in g). Statistically significant differences were determined using ANOVA followed by Students t tests and are denoted by * $p<0.05$ as compared to respective non-stressed controls.

APPENDIX 6.3.3.B.

Open field activity (ambulation, defecation and mobility latency) of BALB/c mice following chronic exposure to predator stress and antidepressant administration (see Figures 6.3.3.ii.1-6).

(A) AMBULATION

WEEK No.	NON-STRESSED CONTROLS			STRESSED		
	VEHICLE	DMI	PAROX	VEHICLE	DMI	PAROX
1 (n=60)	53 ± 4					
3 (n=60)	44 ± 4					
5 (n= 30)	45 ± 5			29 ± 4 *		
7 (n= 30)	39 ± 4			28 ± 3		
9 (n= 7-8)	47 ± 10	52 ± 14	42 ± 6	30 ± 5	38 ± 13	32 ± 5
11 (n= 7-8)	52 ± 8	60 ± 16	51 ± 8	24 ± 5 *	31 ± 7	30 ± 8

(B) MOBIILITY LATENCY

WEEK No.	NON-STRESSED CONTROLS			STRESSED		
	VEHICLE	DMI	PAROX	VEHICLE	DMI	PAROX
1 (n=60)	95 ± 10					
3 (n=60)	115 ± 10					
5 (n= 30)	91 ± 17			164 ± 20 *		
7 (n= 30)	80 ± 15			128 ± 18 *		
9 (n= 7-8)	54 ± 28	63 ± 41	56 ± 14	74 ± 22	84 ± 41	103 ± 36
11 (n= 7-8)	58 ± 24	47 ± 39	49 ± 18	83 ± 24	104 ± 37	67 ± 33

(C) DEFECATION

WEEK No.	NON-STRESSED CONTROLS			STRESSED		
	VEHICLE	DMI	PAROX	VEHICLE	DMI	PAROX
1 (n=60)	5.2 ± 0.2					
3 (n=60)	4.3 ± 0.3					
5 (n= 30)	4.3 ± 0.3			3.4 ± 0.3 *		
7 (n= 30)	4.0 ± 0.4			3.4 ± 0.4		
9 (n= 7-8)	4.9 ± 0.4	3.3 ± 0.6	3.6 ± 0.6	3.3 ± 0.6 *	3.8 ± 0.7	4.5 ± 0.6
11 (n= 7-8)	6.1 ± 0.9	2.9 ± 0.2	4.5 ± 0.3	3.4 ± 0.6 *	2.5 ± 0.5	3.9 ± 0.5

Open field activity (grooming and rearing) of BALB/c mice following chronic exposure to predator stress and antidepressant administration.

(D) GROOMING (data not shown in chapter 6)

WEEK No.	NON-STRESSED CONTROLS			STRESSED		
	VEHICLE	DMI	PAROX	VEHICLE	DMI	PAROX
1 (n=60)	0.4 ± 0.08					
3 (n=60)	0.65 ± 0.1					
5 (n= 30)	1.07 ± 0.63			0.6 ± 0.16		
7 (n= 30)	0.67 ± 0.19			0.6 ± 0.14		
9 (n= 7-8)	0.63 ± 0.3	0.86 ± 0.34	0.5 ± 0.19	0.88 ± 0.4	1.25 ± 0.53	1.75 ± 1.21
11 (n= 7-8)	0.89 ± 0.4	0.25 ± 0.17	0.25 ± 0.25	0.5 ± 0.2	1.25 ± 0.59	0.38 ± 0.18

(E) REARING (data not shown in chapter 6)

WEEK No.	NON-STRESSED CONTROLS			STRESSED		
	VEHICLE	DMI	PAROX	VEHICLE	DMI	PAROX
1 (n=60)	0.65 ± 0.21					
3 (n=60)	0.53 ± 0.17					
5 (n= 30)	0.57 ± 0.16			0.63 ± 0.13		
7 (n= 30)	0.33 ± 0.11			1.1 ± 0.54		
9 (n= 7-8)	0.38 ± 0.26	1.71 ± 0.99	0.38 ± 0.26	0.63 ± 0.32	2.0 ± 1.61	0.88 ± 0.61
11 (n= 7-8)	0.25 ± 0.25	2.75 ± 1.92	0.5 ± 0.19	0.0 ± 0.0	0.25 ± 0.25	0.6 ± 0.27

Open field activity in individually housed mice, recorded before and after the introduction of a predator stress stimulus, see section 6.3.3.ii. Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5 mg/kg) began at the start of week 8 for 28 days. Data are expressed as mean ± sem. A-ambulation scores, B-defecation scores, C-mobility latency, D-grooming scores and E-rearing scores. Statistically significant differences between non-stressed and stressed groups are denoted by $p<0.05$.

APPENDIX 6.3.3.C

Sucrose intake (g) of BALB/c mice following chronic exposure to predator stress and antidepressant administration (see Figures 6.3.3.iii.1/2).

Week no	Test no	NON-STRESSED CONTROLS			STRESSED		
		VEHICLE	DMI	PAROX	VEHICLE	DMI	PAROX
	1 (n=60)	1.11 ± 0.07					
1	2 (n=60)	1.07 ± 0.07					
	3 (n=60)	1.01 ± 0.05					
2	4 (n=30)	0.79 ± 0.04					
	5 (n=30)	1.03 ± 0.08					
3	6 (n= 30)	1.18 ± 0.07			0.89 ± 0.05*		
	7 (n= 30)	1.01 ± 0.04			0.99 ± 0.08		
4	8 (n= 30)	0.6 ± 0.05			0.56 ± 0.08		
	9 (n= 30)	1.06 ± 0.13			0.54 ± 0.06*		
5	10 (n= 8)	1.09 ± 0.07			0.61 ± 0.07*		
	11 (n= 8)						
6	12 (n= 8)	1.35 ± 0.09			0.7 ± 0.09*		
	13 (n= 8)	0.96 ± 0.06			0.63 ± 0.04*		
7	14 (n= 8)	0.74 ± 0.04			0.72 ± 0.05		
	15 (n= 8)	1.17 ± 0.06			0.71 ± 0.06*		
8	16 (n= 8)	0.75 ± 0.11	0.65 ± 0.08	0.59 ± 0.05	0.52 ± 0.06	0.38 ± 0.04*	0.26 ± 0.05*
	17 (n= 8)	0.52 ± 0.05	0.56 ± 0.07	0.58 ± 0.05	0.38 ± 0.07	0.89 ± 0.41	0.48 ± 0.09
9	18 (n= 8)	0.91 ± 0.36	0.66 ± 0.09	0.64 ± 0.04	0.36 ± 0.1	0.59 ± 0.16	0.41 ± 0.13
	19 (n= 8)	0.54 ± 0.08	0.37 ± 0.07	0.54 ± 0.11	0.27 ± 0.05*	0.37 ± 0.1	0.33 ± 0.08
10	20 (n= 8)	0.36 ± 0.07	0.51 ± 0.11	0.70 ± 0.05	0.66 ± 0.1*	0.47 ± 0.09	0.52 ± 0.11
	21 (n= 8)	0.64 ± 0.04	0.81 ± 0.15	0.59 ± 0.03	0.43 ± 0.07*	0.44 ± 0.04*	0.39 ± 0.07*
11	22 (n= 8)	0.86 ± 0.15	0.84± 0.12	0.75± 0.08	0.56 ± 0.05*	0.92 ± 0.36	0.36 ± 0.05*
	23 (n= 8)	0.72 ± 0.14	0.53 ± 0.1	0.69 ± 0.12	0.49 ± 0.08	0.35 ± 0.08	0.45 ± 0.12

Sucrose intakes of individually housed mice were recorded before and after the introduction of a predator stress stimulus, and administration of vehicle, DMI (15 mg/kg) and paroxetine (7.5 mg/kg), see section 6.3.3.iii. Data are expressed as mean ± sem (in g). Statistically significant differences were determined using ANOVA followed by Students t tests and are denoted by * $p<0.05$ as compared to respective non-stressed controls.

APPENDIX 6.3.3.D.

Sucrose intake/g body weight of BALB/c mice following acute exposure to predator stress and antidepressant administration (see Figures 6.3.3.iii.3/4).

WEEK No.	NON-STRESSED CONTROLS			STRESSED		
	VEHICLE	DMI	PAROX	VEHICLE	DMI	PAROX
1 (n=60)	30.4 ± 1.4					
2 (n=60)	35.8 ± 2.4					
3 (n=60)	25.7 ± 1.6					
4 (n= 30)	37.2 ± 2.5			28.5 ± 1.8 *		
5 (n= 30)	18.4 ± 1.5			18.4 ± 3.0		
6 (n= 30)	33.6 ± 2.1			19.7 ± 2.6 *		
7 (n= 30)	29.0 ± 2.0			19.3 ± 1.2 *		
8 (n= 8)	22.9 ± 4	17.9 ± 2.2	18.7 ± 1.9	16.7 ± 2.3	10.9 ± 1.1	8.3 ± 1.8
9 (n= 8)	28.7 ± 11.1	17.7 ± 2.7	21.3 ± 2.2	12.4 ± 3.4	21.3 ± 6.9	14.9 ± 4.8
10 (n= 8)	16.7 ± 2.5	9.6 ± 1.9	17.1 ± 3.2	9.4 ± 1.8 *	11.6 ± 3.0	10.7 ± 2.8
11 (n= 8)	19.4 ± 1.1	21.3 ± 1.5	17.9 ± 0.9	14.2 ± 2.2 *	14.1 ± 1.3 *	12.3 ± 2.3 *
12 (n= 8)	22.0 ± 4.4	14.9 ± 3.5	20.1 ± 3.0	16.4 ± 3.0	11.4 ± 2.6	13.9 ± 3.9

Sucrose intake / g body weight of individually housed mice was calculated for periods before and after the introduction of a predator stress stimulus, see section 6.3.3.3.iii. Administration of distilled water vehicle, DMI (15 mg/kg) and paroxetine (7.5 mg/kg) began at the start of week 8 for 28 days. Data are expressed as mean ± sem (in g). Statistically significant differences were determined using ANOVA followed by Students t tests and are denoted by * $p<0.05$ as compared to respective non-stressed controls.

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